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Protein recruitment to receptor tyrosine kinase-mediated early signalling complexes

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Abstract

Receptor tyrosine kinase (RTK) signalling regulates the activation of numerous cellular processes in response to various external stimuli. Spatio-temporal regulation of protein recruitment to activated tyrosine kinase receptors is important for the generation of specific cellular responses to various external stimuli. The involvement of the signalling proteins Shc, FRS2, Grb2 and Sos and the formation of distinct signalling complexes downstream of three RTKs (TrkA, EGFR, FGFR) was assessed to analyse their role in maintaining signalling specificity. All four signalling proteins played a role in TrkA, EGFR and FGFR signalling, but their recruitment to and involvement in signalling complexes varied depending on the stimulus. The observations indicated that formation of unique multiprotein assemblies provides a mechanism for different receptors to elicit specific signals despite employing the same signalling proteins. Detailed analysis of Shc recruitment to the FGFR2 revealed co-localisation and co-precipitation with the receptor but no direct interaction. This finding provided additional insight into how the availability of binding sites on different receptors regulates the recruitment of individual proteins to receptor-specific signalling complexes.

Secondly, the effects of mutations in the FGFR2 extracellular region on protein recruitment to the receptor and its overall signalling specificity were investigated. Two substitution mutations in the FGFR2, which cause Apert syndrome, result in increased affinity of FGFR2 for FGF. Detailed analysis of the FGFR2 itself and signalling from it in the presence of these mutations indicated that they also result in altered receptor glycosylation, phosphorylation and glycosaminoglycans dependency as well as enhanced Erk1/2 activation. Additionally, recruitment and phosphorylation of Shc were altered in cells expressing the Apert syndrome mutations. The effects of the mutations on the FGFR2 and the signalling complex formed profoundly altered FGFR2-induced signals and cellular responses. These findings highlight the importance of retaining the integrity of protein recruitment and signalling complex formation to achieve signalling specificity.

Declaration

I confirm that the work presented in this thesis is my own. Where information has been derived from other sources, this has been duly indicated within the work.

Annika C. Schüller

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Abbreviations

BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
cDNA	Complimentary DNA
CH1	Collagen homology domain 1
DAG	Diacylglycerol
ddH_2O	Autoclaved distilled water
DMEM	Dulbecco's modified Eagles medium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eGFP	Enhanced green fluorescent protein
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
Erk1/2	Extracellular signal regulated kinases 1 and 2 (also see MAPK)
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FGFR2	Fibroblast growth factor receptor 2 IIIc isoform unless stated
FRS2	Fibroblast growth factor receptor substrate 2
Gab1/Gab2	Grb2-associated binder 1/2
GFP	Green fluorescent protein
Grb2	Growth factor receptor binding protein 2
HEK 293T	Human embryonic kidney cell line
HS	Horse serum
IB	Immunoblot
IP	Immunoprecipitation
IP_3	Inositol(1,4,5)triphosphate
kDa	Kilodalton
MAPK	Mitogen activated protein kinase
M	Molar (moles per litre)
min	Minute
nm	Nanometre

ns	Nanosecond
NGF	Nerve growth factor
OD_x	Optical density (Absorbance at given wavelength x)
PBS	Phosphate buffered saline
PC12	Rat phaeochromocytoma cell line
PCR	Polymerase chain reaction
PGDF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3 kinase
PKB (Akt)	Protein kinase B
PKC	Protein kinase C
PTB	Phosphotyrosine binding (domain)
PtdIns	Phosphatidylinositol
PR/P253R	Pro253Arg substitution in FGFR2
P253R-FGFR2	FGFR2 containing the Pro253Arg substitution, tagged with GFP
RFP/mRFP	Monomoric red fluorescent protein
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH2	Src homology 2 (domain)
SH3	Src homology 3 (domain)
Shc	Src homology and collagen homology containing protein
SNT	Suc1-associated neurotrophic factor target (also see FRS2)
Sos1	Son of sevenless 1
SW/S252W	Ser252Trp subsititution in FGFR2
S252W-FGFR2	FGFR2 containing the Ser252Trp substitution, tagged with GFP
TBS-T	Tris buffered saline containing 0.1% Tween-20 and 0.1M EDTA
TrkA	Tyrosine receptor kinase A/ High affinity NGF receptor
WT	Wild type

Chapter 1

Introduction

1.1 Receptor tyrosine kinase signalling

1.1.1 Signalling from the cell surface

Cell surface receptors are the means by which cells receive specific signals from the outside environment and transmit them to their interior via activation of signalling pathways. The number and types of signals that a given cell can receive and respond to is dependent on the range of receptors it expresses on the cell surface.

1.1.2 Tyrosine kinase receptors

1.1.2.1 Overall molecular structure of receptor tyrosine kinases

The human genome encodes 59 genes for receptor tyrosine kinases (RTKs) that are classed into 20 distinct families on the basis of specific structural differences (Figure 1.1) [1]. All RTKs portray a similar overall structural arrangement: They are single membrane-spanning proteins that contain an often highly glycosylated extracellular ligand-binding region and an intracellular region. The latter consists of the tyrosine kinase moiety and various regions that form docking sites for numerous adaptor proteins.

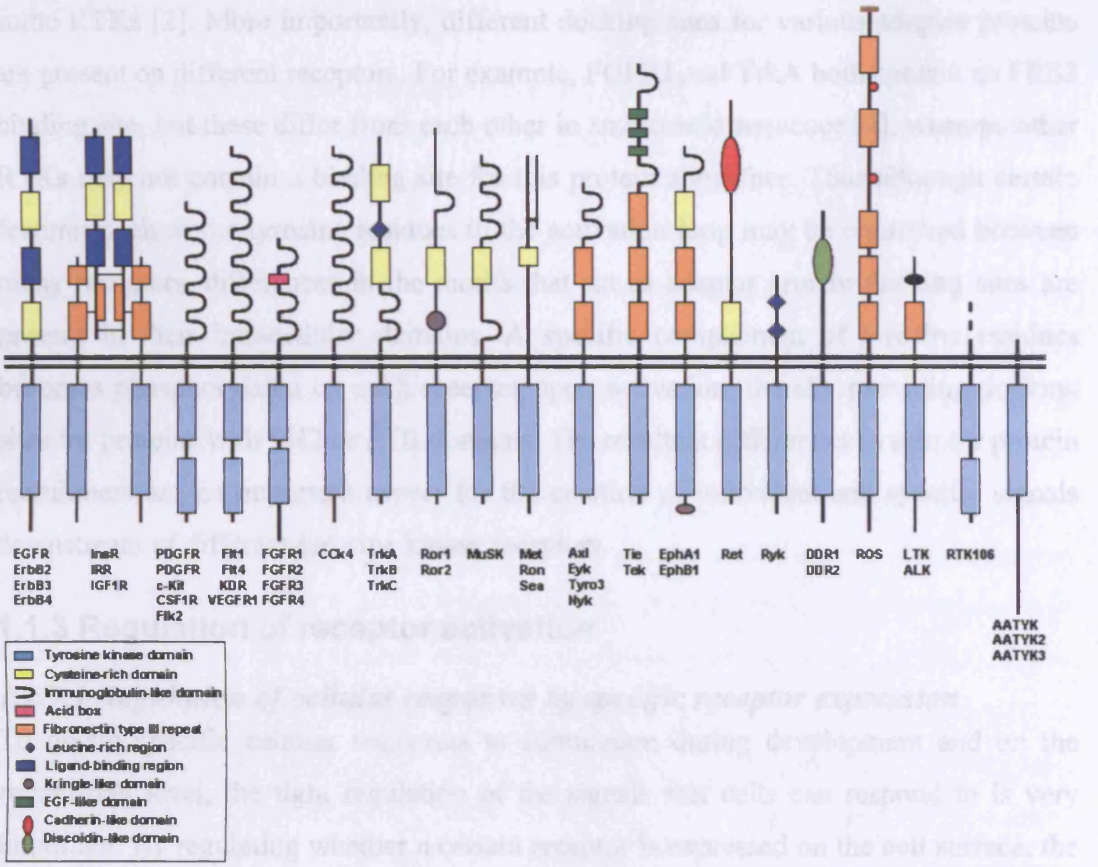


Figure 1.1: Diagrammatic overview of the structural differences between the different receptor tyrosine kinase families Abbreviations of the prototypic receptors: EGFR: epidermal growth factor receptor, InsR: insulin receptor, PDGFR: platelet-derived growth factor receptor, VEGFR: vascular endothelial growth factor receptor, FGFR: fibroblast growth factor receptor, CCk: colon carcinoma kinase, Trk: tyrosine receptor kinase, Ror: receptor orphan, MuSK: muscle-specific kinase, Tie: tyrosine kinase receptor in endothelial cells, EphR: ephrin receptor, Ret: rearranged during transfection, Ryk: receptor related to tyrosine kinases, DDR: discoidin domain receptor, ROS: receptor tyrosine kinase expressed in some epithelial cell types, LTK: leukocyte tyrosine kinase. Adapted from [2, 3].

1.1.2.2 Differences between individual RTKs

The different RTKs vary mostly in the composition of the extracellular region. The assembly of recurring motifs in different combinations and orders gives rise to a number of different receptors (Figure 1.1). The reason for the greatest variation being found in this region is obvious when considering that each receptor has to be exactly matched to bind only a specific type of ligand. The different domains assemble to form specific binding pockets that allow recognition of a diverse range of ligands that bind to the receptors [2].

Further differences between RTKs are present in the intracellular region, such as for example the presence and length of kinase insert that results in a split-kinase domain in

some RTKs [2]. More importantly, different docking sites for various adaptor proteins are present on different receptors. For example, FGFR1 and TrkA both contain an FRS2 binding site, but these differ from each other in amino acid sequence [4], whereas other RTKs may not contain a binding site for this protein altogether. Thus although certain features such as the tyrosine residues in the activation loop may be conserved between many receptors, differences in the motifs that act as adaptor protein docking sites are present in their intracellular domains. A specific complement of tyrosine residues becomes phosphorylated on each receptor upon activation, thereby providing docking sites for proteins with SH2 or PTB domains. The resultant differences in adaptor protein recruitment are an important aspect for the creation of individual and specific signals downstream of different tyrosine kinase receptors.

1.1.3 Regulation of receptor activation

1.1.3.1 Regulation of cellular responses by specific receptor expression

To obtain specific cellular responses to stimulation during development and on the organismal level, the tight regulation of the signals that cells can respond to is very important. By regulating whether a certain receptor is expressed on the cell surface, the response to specific extracellular signals can be controlled, which is of particular relevance during development. Additionally, specific expression patterns of individual receptors as well as individual receptor isoforms are important in regulating tissue-specific responses to growth factors.

1.1.3.2 Availability of different ligands for RTKs

In addition to cell- or tissue-specific expression of receptor families and isoforms, receptor tyrosine kinase signalling is regulated by the existence of several ligands and their specific expression patterns. PDGFα and PDGFβ (platelet-derived growth factors) can form homo- or hetero-dimers that are linked by disulphide bridges. The type of dimer determines which specific combination receptors (i.e. PDGF receptor α/α, β/β or α/β dimers) can be activated. The epidermal growth factor receptor (EGFR) family can be activated by a number of different ligands, including EGF, TGFα and various neuregulins. Similarly, the 23 different fibroblast growth factors (FGFs) discovered to date have been shown to exhibit a certain degree of specificity for the different isoforms of the four FGF receptor subgroups [5-8]. Thus by regulating both the expression of receptors and ligands, tight control over cellular responsiveness to stimulation of different receptor pathways can be exerted.

1.1.4 Activation of RTKs by ligand binding

1.1.4.1 Formation of ligand-receptor complexes

With the exception of the insulin receptor, engagement of a tyrosine kinase receptor by its respective ligand leads to dimerisation followed by activation of the kinase moiety and *trans*-autophosphorylation on tyrosine residues. Depending on the ligand, active receptor dimers are formed in various ways. Growth factors such as the vascular endothelial growth factor (VEGF) exist as homodimers, which bind to their respective receptors in a 2:2 complex. In the case of the EGFR, a 2:2 complex is formed, but receptor dimerisation is only driven by receptor-receptor interactions and no ligand-ligand interactions. The fibroblast growth factor (FGF) also binds to its receptor in a 2:2 fashion, but the FGF-FGFR interactions alone are not sufficient to stabilise dimer formation. The stable formation of receptor dimers is only achieved in the presence of glycosaminoglycans (GAGs) such as heparan sulphate proteoglycans (HSPGs). These molecules allow further regulation of the specific activation of the FGFR (reviewed in [9] and refer to section 1.4.2.2). Thus depending on the ligand and the receptor, different types of receptor/ligand dimers are formed. This is important for productive RTK activation and signalling and also contributes to regulation of specific receptor activation in different cellular contexts.

1.1.4.2 Formation of larger multimeric structures

Although the model of receptor dimerisation upon ligand binding is still widely accepted, more recent studies have indicated that receptor dimers may also form in the absence of ligand (reviewed in [10, 11]). In the unstimulated state, receptor monomers are in equilibrium with receptor dimers, but only a very small proportion would be found in the active dimer state in which the quaternary structure is such to allow kinase activation and *trans*-autophosphorylation. Ligand binding to the receptor causes stabilisation of the dimer and transition into the active dimer state, which leads to full activation and downstream signalling. In addition, evidence exists that receptor dimers are only the smallest possible functional unit and that the formation of larger complexes is possible, leading to receptor aggregation in certain regions of the plasma membrane [12, 13].

1.1.4.3 Intracellular effects of ligand binding

The ligand binding event is transmitted to the kinase domain and *trans*-autophosphorylation of two subunits in a receptor dimer on numerous tyrosine residues takes place. The location of these tyrosine residues and their presence within different

amino acid motifs is specific for each receptor. Kinase activity is often regulated by the presence of activation loops which contain tyrosine residues that need to be phosphorylated first in order for the kinase to reach full activity (reviewed in [14]).
Using the FGFR1 as a model, its has recently been shown that all phosphorylation events occur in a highly ordered, sequential manner [15]. Additionally, the phosphorylation of Y653 and Y654 in the activation loop led to 50-100 fold and 500-1000 fold increase in kinase activity respectively. These findings may be representative of the way in which most RTKs function and indicate that phosphorylation of the various tyrosine residues does not occur in a random fashion. Such a mechanism adds further temporal regulation to the recruitment of proteins and the activation of downstream pathways.

1.1.5 Recruitment of adaptor proteins to activated RTKs

Recruitment of signalling adaptors such as FRS2, Shc and Grb2 to the activated and phosphorylated RTKs occurs primarily via interaction of SH2 or PTB domains with phosphotyrosine residues/motifs on the receptor. Once recruited, these signalling proteins may be phosphorylated on tyrosine residues themselves or form platforms for the recruitment of other proteins. Recruitment of enzymes such as for example PLCγ1, PI3K, or Akt can lead to activation by phosphorylation, conformational change or simple recruitment to the membrane respectively. This allows recruitment of other signalling proteins via secondary interactions as well as formation of secondary messengers which play important roles in downstream signalling. Overall, a variety of proteins are recruited directly to the activated receptor or its vicinity via multiple protein-protein interactions mediated by a range of modular domains. The implications and importance of specific recruitment of proteins and activation of signalling proteins will be described in more detail in later sections.

1.1.6 Cellular roles of RTKs and activation of downstream targets

1.1.6.1 Overall cellular responses regulated by RTK signalling

RTKs are involved in the activation of a number of different cellular processes. These range from developmental control, cell division and regulation of the cell cycle to migration and alteration of cell shape, differentiation, apoptosis and cell metabolism. The specific responses to activation of any given receptor are often cell type specific. For example, activation of the fibroblast growth factor receptor leads to neurite outgrowth and differentiation in PC12 cells but to proliferation in NIH3T3 cells [16].

Thus cellular context and specific expression patterns of certain types of receptors are important in the regulation of downstream responses to RTK activation in vivo. The following sections briefly outline the activation of the three major signalling pathways activated downstream of RTKs.

1.1.6.2 The Ras/Raf/MEK/Erk1/2 pathway

Activation of RTKs leads to the recruitment of Grb2/Sos1 complexes to the plasma membrane via interaction of the Grb2 SH2 domain with the RTK itself or other tyrosine phosphorylated adaptor proteins such as Shc or FRS2. Grb2 interacts with a proline-rich region in the C-terminus of Sos1 (from hereon referred to as Sos) via its two SH3 domains [17]. Once in proximity at the plasma membrane, Sos acts as a guanine nucleotide exchange factor for the small G-protein Ras (which is itself constitutively membrane anchored via a farnesyl moiety [18]). The activated, GTP-bound Ras interacts with and recruits members of the Raf kinase family, which are subsequently activated by highly regulated dephosphorylation and phosphorylation events. Raf is able to phosphorylate the MAPK/Erk kinase1 (MEK1, referred to as MEK), which in turn is activated by this phosphorylation event. MEK is a dual kinase that subsequently phosphorylates Erk1/2 (extracellular signal regulated kinase, also known as mitogen activated protein kinase, MAPK) on threonine and tyrosine residues. Activated Erk1/2 can phosphorylate and interact with various cytoplasmic targets, but is also able to translocate to the nucleus. Here it is involved in regulation of gene transcription by phosphorylating various transcription factors such as STAT3 and Elk-1 as well as kinases such as p90RSK (reviewed in [19-21]).

1.1.6.3 Activation of the phosphatidylinositol-3-kinase (PI3K) pathway

The PI3K pathway is most strongly activated in response to stimulation of the insulin receptor but is also activated in response to a number of other RTKs. The SH2 domain of the p85 regulatory subunit of PI3K allows binding to phosphotyrosine motifs on the receptors or on adaptor proteins such as IRS1 or Gab1 [22-27]. The conformational change induced in the p110 catalytical subunit as a result of this binding event leads to PI3K activation. PI3K phosphorylates the D-3 position of phosphatidylinositol(4)phosphate and phosphatidylinositol(4,5)bisphosphate (PtdIns(4)P) and PtdIns(4,5)P_2 respectively) to create second messengers that lead to membrane recruitment of proteins via their PH domains. Of particular importance is the generation of PtdIns(3,4,5)P_3, which leads to recruitment and activation of the protein kinase B (PKB or Akt) and phosphatidylinositol-dependent kinase1 (PDK1). These

serine-threonine kinases are involved in signal relay to other effector proteins such as the kinases GSK3 and p70S6K, transcription factors like FKHR-L1 or survival regulators such as BAD. Other non-receptor protein kinases and adaptor proteins containing PH domains can also be recruited to the membrane as a result of the creation of these second messengers in the membrane. The PI3K pathway is important in regulating cell survival, growth, migration, cell cycle entry and nutrient sensing (reviewed in [28]).

1.1.6.4 The role of phospholipase Cγ-1

Phospholipase Cγ-1 (referred to as PLCγ from hereon) can directly bind to phosphotyrosine sites on RTKs via its SH2 domain. It becomes activated by tyrosine phosphorylation and subsequently cleaves the membrane lipid $Ptd(4,5)P_2$ to produce the two second messengers diacylglycerol (DAG) and inositol-(1,4,5)-phosphate (IP3). Whereas DAG is involved in activation of the protein kinase C (PKC) and subsequent pathways downstream of this kinase, IP3 triggers the release of calcium from the endoplasmic reticulum. The released calcium binds to calmodulin which causes activation of calmodulin-dependent kinases and is also involved in leading to complete activation of PKC. Activated PKC can also feed into the Erk1/2 pathway by causing phosphorylation of Raf [29]. By activation of numerous pathways via the two different second messengers, PLCγ regulates various intracellular signalling events and is involved in activation of various transcription factors (reviewed in [9, 30]).

1.2 Epidermal growth factor receptor signalling

1.2.1 ErbB receptors and their ligands

1.2.1.1 ErbB receptor family

The epidermal growth factor receptor (EGFR) was the first tyrosine kinase receptor discovered in 1978 [31]. Since then, four genes encoding ErbB1 (EGFR), ErbB2 (HER2), ErbB3 and ErbB4 have been discovered in mammals. ErbB4 is additionally subject to alternative splicing, which leads to expression of four different variants of this receptor. The ErbB receptors can homo- or heterodimerise. The latter property is particularly important in the case of ErbB3 and ErbB2, which lack kinase activity and ligand-binding ability respectively (reviewed in [32]).

1.2.1.2 ErbB receptor ligands

Eleven ligands with the ability to interact with the ErbB receptor family have been described, including EGF, heparin-binding EGF (HB-EGF), transforming growth factor alpha (TGFα), betacellulin, amphiregulin, epiregulin, epigen and the neuregulins (NRGs) 1-4 [33]. Whereas some of these ligands are specific to one type of ErbB receptor (for example EGF binds only to EGFR/ErbB1), others such as NRG1 and NRG2 can bind to different ErbB receptors. Several of the ligands are initially expressed as membrane anchored proteins and need to be released by proteolytic cleavage, whereas others are bound to cell surface proteoglycans, which thereby provide a reservoir from where ligand can be made available for receptor activation (reviewed in [34]). Such differences may be important in regulation of activation of different ErbB receptors in various cellular contexts.

1.2.2 Structure and activation

1.2.2.1 ErbB structure

Like other RTKs, the ErbB receptors are made up of an extracellular region, a single transmembrane domain and an intracellular region containing the kinase domain and autophosphorylation sites that allow adaptor protein binding. The extracellular domain can effectively be divided into four subdomains, two homologous cysteine-rich regions (S1 and S2) and two regions that form the ligand binding site (L1 and L2) (Figure 1.1). The cysteine residues in the S1 and S2 domains do not form disulphide bridges. The structure of the extracellular domain of the EGFR in the presence of EGF and TGFα has been solved and revealed that the interaction of the L1 and L2 regions creates the ligand binding pocket [35, 36].

1.2.2.2 Ligand binding

The crystal structures of the EGFR with ligand revealed an important role of the S1 region in receptor dimerisation [35, 36]. Each S1 domain projects out a 'dimerisation loop', which are thought to interact with each other via interactions that stabilise dimer formation. Receptor dimers have been shown to have both greater stability and ligand-binding affinity than the respective monomers [37].

1.2.2.3 ErbB homo- and hetero-dimerisation and receptor activation

In the case of the different receptors found in the ErbB family, it has been demonstrated that hetero-oligomerisation can occur. In particular, the ErbB2 receptor, for which no ligand has been described to date, can oligomerise with the EGFR, leading to its

activation [38, 39]. Other members of this family have since been found to form hetero-oligomers and this may be an important aspect in providing further control over the downstream signalling pathways that can be activated in particular cells [40, 41].

The EGFR can exist in a dimeric state in the absence of EGF, but the addition of the growth factor shifts the equilibrium from a monomeric to a dimeric state [12, 42]. EGF and the EGFR form 2:2 complexes upon dimerisation. This dimerisation is not ligand-dependent, and occurs purely on the basis of receptor-receptor interactions (i.e. no EGF:EGF contacts are made) [35]. EGF:EGFR dimer formation is regulated by interaction of the dimerisation loop of the C1 domain with the other receptor [35, 43]. In addition to the dimerisation loop, contacts mediated by the helical transmembrane domains may also contribute to the dimer formation. The transmembrane region regulates aspects such as receptor association and spatial arrangement, which subsequently influence downstream signal output (reviewed in [32]).

In contrast to many other RTKs, the activation loop of the EGFR does not need to be phosphorylated to assume the active conformation, so that the EGFR can be active in the absence of ligand [44]. The addition of EGF however is important to allow conformational changes to take place that normally act to prevent dimerisation of the extracellular domain in the absence of ligand [45]. Dimerisation of the intracellular domain and correct positioning of the kinase domain to allow trans-autophosphorylation seem to be sufficient to allow complete activation of the receptor and subsequent recruitment of various signalling proteins [46].

1.2.3 Signalling downstream of the EGFR

Once dimerised and activated by the addition of EGF ligand, the EGFR is phosphorylated on ten tyrosine residues (Y845, Y891, Y920, Y992, Y1045, Y1068, Y1086, Y1114, Y1148 and Y1173), not all of which are required for recruitment of signalling proteins such as Shc, Grb2 or PLCγ (reviewed in [33]). Instead of having a single, specific binding site, several of these proteins are able to bind multiple sites on the EGFR. In comparison with other RTKs, it also interesting to note that all of the tyrosine residues are located in the very C-terminal region of the intracellular domain. Figure 1.2 provides a simplified diagram of the main pathways activated by the EGFR and the roles of the signalling proteins relevant to this work are discussed below.

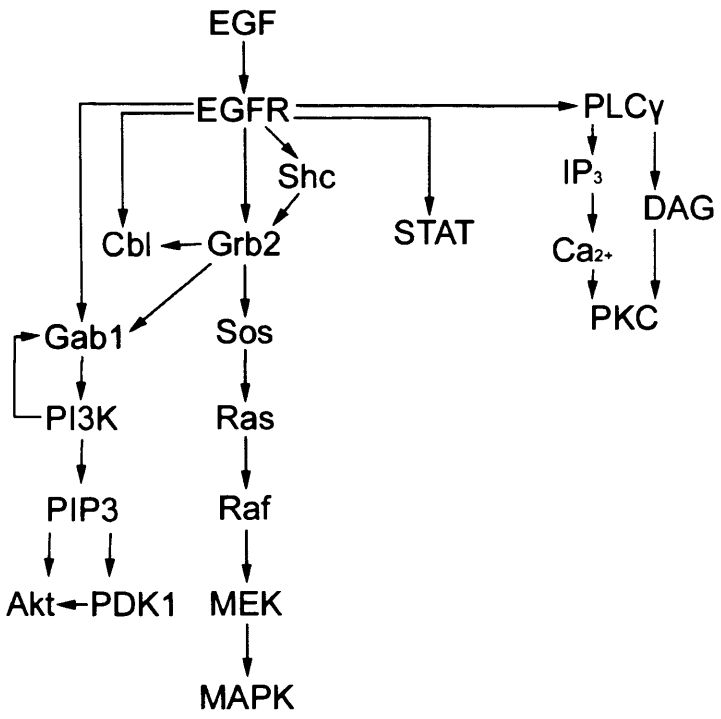


Figure 1.2: Overview of the main signalling pathways initiated by the EGFR The proteins involved in signalling from the EGFR and the activation of the three main downstream signalling pathways are indicated. Negative-feedback signalling pathways are not shown. Adapted from [47].

1.2.3.1 Grb2

The SH2/SH3 domain-containing adaptor protein Grb2 (growth factor receptor bound protein 2) can bind directly to the EGFR at various sites through interaction of its SH2 domain with phosphotyrosine motifs surrounding Y1068 (major site) and Y1086 (minor site) [48]. By recruitment to the membrane, Grb2 leads to translocation of Sos to the plasma membrane.

1.2.3.2 Shc

The Src homology and collagen containing protein (Shc) adaptor binds to the EGFR at two sites, namely Y1173 (major site) and Y992 (minor site) in response to EGF stimulation, although other sites are able to compensate for the loss of these binding sites for Shc [48]. Shc interacts with the EGFR via its N-terminal PTB domain or its C-terminal SH2 domain [49, 50]. However, it can still be tyrosine phosphorylated in the absence of the main EGFR autophosphorylation sites, so receptor phosphorylation is not directly coupled to Shc activation [51, 52]. Once phosphorylated, Shc provides further binding sites for the Grb2/Sos complex, which act in conjunction with the direct Grb2 binding sites on the EGFR.

1.2.3.3 FRS2

The fibroblast growth factor receptor substrate 2 (FRS2) is a myristoylated adaptor protein that is tyrosine phosphorylated in response to TrkA and FGFR activation and acts as a docking site for the recruitment of proteins to the plasma membrane [53, 54]. A recent report has implicated FRS2 with a possible role in EGFR signalling, since it was observed to bind directly to the EGFR and has been suggested to play a role in Erk1/2 activation. Moreover, it can be serine/threonine phosphorylated by Erk1/2 in response to EGF stimulation, which indicates negative feedback via the pathway that it activates [55]. In addition, FRS3 (also known as FRS2β) plays a role in negative regulation of Erk2 in the absence of great levels of tyrosine phosphorylation in response to EGF [56]. No other studies revealing a direct role for FRS2 in EGFR signalling have been reported, and its exact role downstream of the EGFR needs to be elucidated further.

1.3 TrkA signalling

1.3.1 Neurotrophin receptors and their ligands

1.3.1.1 Neurotrophins and their receptors

To date three different receptor tyrosine kinases have been identified in vertebrates that act as receptors for neurotrophins: TrkA, B and C. Although structurally similar, the three receptors portray selectivity for ligands. TrkA is the high affinity receptor for NGF, which is to be investigated in this work. In addition, $p75^{NTR}$ is another transmembrane receptor that is able to bind NGF. In contrast to the neuron-survival signal initiated by NGF binding to TrkA, $p75^{NTR}$ activates signalling pathways that promote neuronal cell death.

1.3.1.2 Neurotrophins

NGF was the first of the neurotrophins to be discovered. Six members of the neurotrophin family have been discovered to date: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-cell-line-derived neurotrophic factor (GDNF), neurturin, and the neurotrophins NT-3, 4 and 5. NGF and BDNF are ligands for TrkA, whereas NT-4 and NT-5 bind specifically to TrkB and NT-3 is the specific ligand for TrkC. GDNF interacts with the RET receptor and the GDNFRα, a co-receptor lacking a cytosolic domain that is required for RET activation. Thus as observed for other RTKs, the selectivity in terms of ligand is an important regulator of receptor activity in different cellular environments.

1.3.2 Physiological functions of neurotrophin signalling

The work presented in this thesis will focus on TrkA and its activation by NGF, therefore signalling from this receptor will be described in more detail. The different neurotrophin receptors show restrictive expression patterns but all are mostly expressed in brain and other neural tissues. Expression in particular cellular contexts contributes to specific functions of different neurotrophin receptors. This is important for the mediation of various neurotrophin-mediated cellular events such as apoptosis, survival and differentiation [57].

TrkA has been found to co-precipitate with $p75^{NTR}$. This has opened up the possibility that these two receptors interact and that this is important for the regulation of the signals emanated from these receptors. It has been proposed that only the interaction of $p75^{NTR}$ with TrkA leads to the formation of high affinity binding sites for NGF [58], although more recently independent functions for $p75^{NTR}$ have been described [59, 60].

Several steps are required for neuronal differentiation to occur, including cell migration, directional axonal growth, synaptogenesis and selective survival. All of these processes are stimulated or activated through various signalling pathways. Signalling in response to neurotrophin stimulation plays an important role in regulating neuronal differentiation, in addition to the signals emanated by cell adhesion molecules [61]. Whereas $p75^{NTR}$ is involved in regulation of a variety of functions such as organ development, germ cell maturation and testicular cell differentiation (reviewed in [62]), Trk receptors are the main mediators of neurotrophin signalling in developing and adult neurons. In addition, NGF has also been shown to carry out important functions in non-neuronal cells such as acting as an autocrine survival factor in memory B cells [63]. The investigation of the signalling events from TrkA investigated in this work will focus on its activation of the Erk1/2 pathway, which is an important regulator of differentiation.

1.3.3 Structural aspects of TrkA signalling

The extracellular region of TrkA consists of five domains, namely two cysteine-rich regions (domains 1 and 3), three leucine-rich repeats (domain 2), and two immunoglobulin-like domains (domains 4 and 5) (Figure 1.1). Although all Trk receptors share this domain assembly, the extracellular regions of the three receptors only portray 50 to 55% sequence homology [64]. The Ig-like domains are sufficient for NGF binding. Whereas domain 5 is required for efficient interaction of TrkA with its ligand and single amino acids important in NGF binding have been mapped to the EF loop of domain 5, domain 4 is required for correct folding of domain 5 and is therefore

important in regulation of ligand binding (reviewed in [65]). In addition, the Ig-like domains have been implicated with a role in prevention of receptor dimer formation in the absence of ligand, and are therefore important regulators of the activation of the receptor as well as downstream signalling.

The NGF dimer interacts with the TrkA dimer (two copies of domain 5) via its central β-sheet region. The residues on NGF important for interaction with $p75^{NTR}$ remain partially exposed, which allows formation of a complex involving TrkA, $p75^{NTR}$ and NGF [66]. Specificity is introduced into the TrkA-NGF interaction by the existence of a 'specificity patch', which contains a low level of sequence homology between the neurotrophins as well as the Trk receptors. Although the neurotrophin binding pocket is conserved between the receptors, the residues within it vary greatly, which allows specific recognition of the different types of ligands. In addition, a second patch, the 'conserved patch', is important in ligand-receptor interaction. In contrast to the 'specificity patch', the residues within this region are highly conserved between the Trk receptors and contact is made by the receptor with both NGF molecules instead of a single ligand molecule [66].

1.3.4 Signalling downstream of TrkA receptor

Activation of TrkA by binding of NGF leads to the recruitment of numerous signalling proteins. Because of its importance in neuronal differentiation, TrkA activates various pathways. The correct interplay between these pathways is important for the activation of the correct cellular response to NGF. Only the main proteins recruited to and phosphorylated/activated by TrkA that are relevant to the studies presented in Chapter 3 will be discussed in this section (refer to Figure 1.3 for diagrammatic representation).

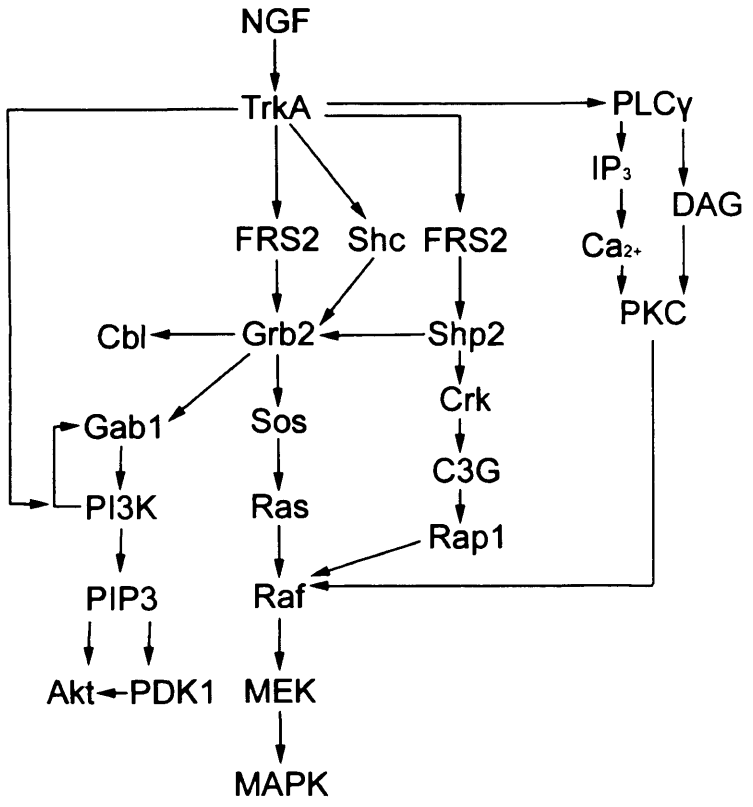


Figure 1.3: Overview of the main signalling pathways initiated by TrkA The proteins involved in signalling from TrkA and the activation of the three main downstream signalling pathways are indicated. Negative-feedback signalling pathways are not shown. Adapted from [67].

1.3.4.1 Grb2

Grb2 can be recruited to the TrkA receptor via phosphorylated FRS2 or Shc, but has recently been shown to also interact with TrkA directly [68]. Three sites for Grb2 binding were identified, namely the two tyrosines in the activation loop (Y683 and Y684) and Y794. Binding to the activation loop tyrosines may be important in keeping the receptor activated (i.e. prevent dephosphorylation by phosphatases). This indicates a role for Grb2 in addition to Sos recruitment to the plasma membrane.

1.3.4.2 Shc

Shc binds to the same site on TrkA as FRS2 via its PTB domain [69-71]. Once phosphorylated, Shc recruits the Grb2/Sos complex. This process has been shown to be required for NGF induced differentiation of PC12 cells [72].

1.3.4.2 FRS2

Exposure to NGF causes tyrosine phosphorylation of Y490 on TrkA, which is embedded in a canonical PTB domain binding NPXpY sequence [4]. Following phosphorylation, this site forms a binding site for the PTB domain of the membrane

anchored adaptor protein FRS2. FRS2 competes with Shc for this binding site and it has been proposed that this competition provides a mechanism to differentiate between activation of transient and prolonged Erk1/2 signalling in PC12 cells [70]. Inhibition of the PLCγ signalling pathway almost completely abolishes FRS2 phosphorylation in response to NGF stimulation of TrkA [73]. In addition to recruitment of the Grb2/Sos complex, FRS2 is further involved in the recruitment of Crk and subsequently the guanine nucleotide exchange factor for Rap1, C3G, to the plasma membrane [67].

1.4 Fibroblast growth factor signalling

1.4.1 The FGFR family

1.4.1.1 FGFR domain composition

The extracellular region of the FGFR comprises three Ig-like domains (D1, D2 and D3) and a stretch of seven to eight acidic residues between D1 and D2 referred to as the acid box (Figure 1.1). The FGFR contains a split-kinase domain and numerous tyrosine residues in the cytoplasmic region that become phosphorylated upon activation.

1.4.1.2 Subfamilies, isoforms and alternative splicing

Four FGFR genes (*fgfr1-4*) with great sequence similarity have been identified [74, 75]. The *fgfr-1-3* can all be expressed as different isoforms as a result of alternative splicing, whereas the FGFR4 exists only as a single isoform. Alternative splicing may result in the expression of FGFRs with or without the Ig-like D1 domain [76]. The lack of the D1 domain increases affinity of the receptor towards both FGF and heparin [77, 78]. Most importantly, alternative use of exons encoding the second part of the D3 domain is an important determinant of specificity in the FGF-FGFR interaction, because the D3 domain (along with the D2 domain and the D2-D3 linker region) is involved in FGF binding [79, 80]. Restricted expression of the IIIb isoform in epithelial cells and of the IIIc isoform in mesenchymal cells invokes additional specificity to FGFR signalling [81].

1.4.2 FGFR ligands

1.4.2.1 Fibroblast growth factors

22 human FGFs have been described to date [82]. Although FGF23 exists, no human FGF15 has been described, and human FGF19 is believed to be homologous to mouse FGF15 [83]. FGFs are classed into sub-families, within which a greater amount of

sequence homology, similar expression patterns and receptor binding properties are observed. Some of the residues that make contact with the FGFR are found within an internal core region made up of 28 highly conserved and six identical amino acids found within most FGFs. The primary heparin binding site on FGF molecules is distinct from the FGFR binding site [84, 85].

1.4.2.2 Heparan sulphate proteoglycans

Heparan sulphate proteoglycans (HSPG) are required for the interaction of FGF with the FGFR [86]. The structure of the heparin-FGF-FGFR complex has been solved by different groups and two different models for the formation of heparin-FGF-FGFR complexes were proposed (reviewed in [87]). The first model (proposed by Pellegrini *et al.*) proposes dimerisation of the FGFs by heparin in the absence of any FGF-FGFR interactions, which results in a 1:2:2 heparin:FGF:FGFR complex [88]. In contrast, the Schlessinger/Mohammadi model proposes formation of a 2:2:2 complex: FGF and FGFR make complex with the respective other member of the dimer, the heparin molecules reduce the electrostatic repulsion between them [89]. However, the addition of excess heparin did not alter the 1:2:2 complex described by Pellegrini *et al.*, and the addition of over 10-fold excess heparin to FGF1 still only led to the formation of 1:2 heparin:FGF1 complexes [90, 91]. Additionally, several molecules of FGF can assemble on a long polysaccharide molecule, which would favour a model in which clustering of receptors around few glycosaminoglycans chains occurs [90].

The 'two-end-model' was formulated taking these observations into account. It suggests that upon FGF-FGFR binding, the heparin binding sites on the two FGFs and the two D2 domains of the receptors in a dimer come together to form a 'canyon' in which a single HSPG molecule can bind. Since each FGF will contribute slightly differently to the binding site, this model can also explain how specificity and preference for certain HSPGs is achieved despite the D2 domain being the same in both the IIIb and IIIc isoforms. In addition, the mode of binding in this model is highly cooperative, and involves contacts of FGF and FGFR with heparan sulphate (HS), as well as ligand-receptor and receptor-receptor interactions that are facilitated by HS binding [87].

1.4.3 Specificity in FGFR signalling

Some degree of redundancy and promiscuity in FGF-FGFR binding exists, since knockout mice of many FGFs are viable or have mild phenotypes (reviewed in [92]). Nonetheless, certain FGF-FGFR interactions are very specific. The binding affinities of individual FGFs for the various FGFR isoforms have been assessed in two different

ways. Mohammadi *et al.* expressed the D2 and D3 ligand binding region of the FGFRs bacterially and measured the affinity for FGFs using surface plasmon resonance [7, 8]. In contrast, Ornitz *et al.* used cellular based assays measuring the mitogenic activity of different FGFRs in response to various FGFs [5, 6]. The different approaches may explain some of the differences observed in the relative binding affinities reported. However, the overall pattern reported by both groups is similar and indicates that there is some selectivity in FGF-FGFR binding. For example, FGF1 binds to all FGFRs to a similar extent, whereas FGF7 shows a great deal of specificity for FGFR2IIIb and does not bind significantly to other FGF receptors.

The specificity in ligand recognition is mostly achieved by alternative splicing creating the alternative D3 domains, which makes the most contact with the FGF molecule in the reported structures. Additional specificity in the FGF-FGFR interaction is achieved by the presence of specific glycosaminoglycans (GAGs). Differences in the type, length and sulphation pattern of proteoglycans are important to achieve maximal binding of different FGFs to their receptors [93-96]. Different requirements were observed even if either the FGF or the FGFR were kept the same, which indicates that various GAGs can introduce further specificity into FGFR activation and signalling [97].

Additionally, highly regulated expression of both ligands and receptors allows controlled and specific activation of receptors to take place [92]. For example, the mesenchymally expressed FGF7 and FGF10 can only activate the epithelial FGFR2IIIb isoform, whereas epithelial FGFs 2, 4, 6, 8, 9 and 17 bind mesenchymally expressed FGFR2IIIc with higher affinity to avoid autocrine signalling [5, 98, 99].

1.4.4 Signalling downstream of the FGFR

FGFR signalling is important in regulation of a multitude of cellular functions such as apoptosis, proliferation, migration, differentiation and survival (reviewed in [75]). The involvement of FGFR signalling in development and particularly limb formation plays an important role in the development of various skeletal diseases (as discussed in section 1.6).

Most studies on FGFR signalling have been carried out using the FGFR1 and it is widely accepted that there is not much difference in signalling pathways initiated by different receptor types and isoforms due to the high degree of sequence homology in the intracellular region [75]. Indeed, an analysis of the overall tyrosine phosphorylation of proteins in response to receptor activation by Raffioni *et al.* seemed to reveal that the only difference between FGFR1, 3 and 4 was the level of phosphorylation but not the

type of proteins that were phosphorylated [100]. This finding was mostly supported by the observation that activation of PLCγ and Erk1/2 was weaker in cells expressing the FGFR4 than the FGFR1 [101]. However, this study also reported an altered activation of transcription factors in response to activation of the two different FGFRs and altered intracellular trafficking of the four different FGFRs has also been described [102]. Thus differences between various FGFRs do seem to exist.

Since most studies focusing on signal transduction from the activated FGFR have employed FGFR1, the following descriptions may not necessarily hold true for all receptor isoforms. The FGFR1 is autophosphorylated on seven tyrosine residues (Y463, Y583, Y585, Y653, Y654, Y730, Y766), of which Y653 and Y654 are positioned in the activation loop and are responsible for autoinhibition. Phosphorylation of all but Y766 is not required for activation of Erk1/2 [103]. Autophosphorylation of these residues occurs in a highly controlled, sequentially manner, which may be important for spatial and temporal signalling downstream of the receptor [15]. Figure 1.4 outlines the major signalling pathways activated by the FGFR and the roles of the proteins relevant to this study as well as several other FGFR effectors are described in more detail below.

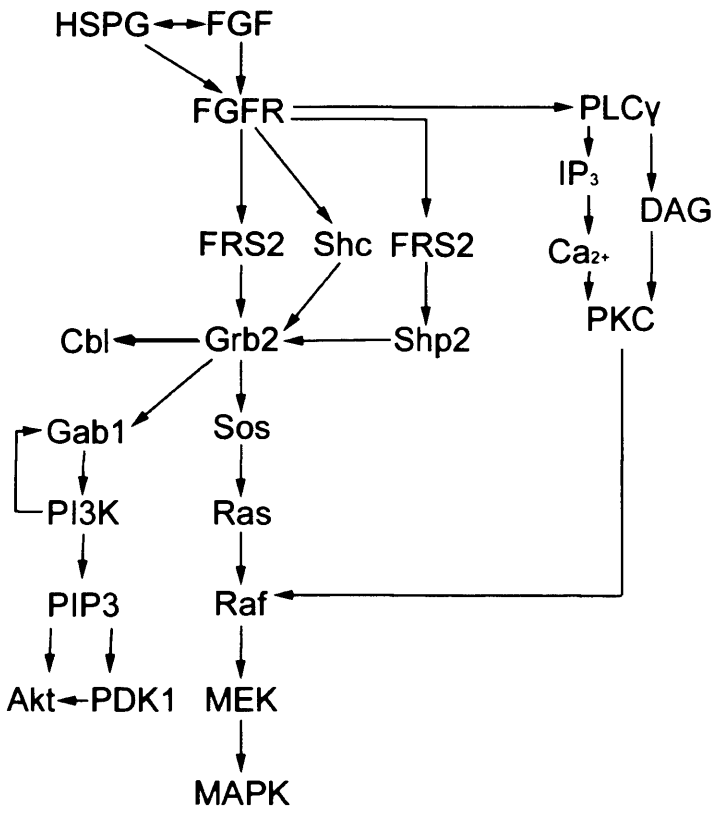


Figure 1.4: Overview of the main signalling pathways initiated by the FGFR The proteins involved in signalling from the FGFR and the activation of the three main downstream signalling pathways are indicated. Negative-feedback signalling pathways are not shown. Adapted from [47].

1.4.4.1 Grb2

Although Grb2 has been shown to bind to RTKs like the EGFR directly via its SH2 domain, such an interaction could not be detected in the case of the FGFR [104]. Thus the recruitment of the Grb2/Sos complex to the FGFR is mediated by indirect mechanisms via interaction of Grb2 with tyrosine phosphorylated sites on Shc, FRS2 and Shp2.

1.4.4.2 Shc

Although its involvement in FGFR signalling is not entirely clear, Shc is phosphorylated and associates with Grb2 in response to FGFR activation [101, 104]. Shc phosphorylation is not dependent on receptor phosphorylation on residues other than Y653 and Y654, which are required for kinase activity [103]. Although many studies failed to observe interaction of Shc with the FGFR1, Curto *et al.* showed that Shc and the FGFR1 could be co-precipitated in v-Src transformed cells [105], which indicates a potential role of Shc in FGFR signalling. Further, Shc has been found to co-precipitate with the FGFR3 [106].

1.4.4.3 FRS2

FRS2 plays a major role in FGFR signalling, as demonstrated by the fact that FRS2-/- mouse embryos die at E7-7.5 [53, 107]. FRS2 binds constitutively to the FGFR1 via its PTB domain and is heavily tyrosine phosphorylated upon FGFR activation [108]. Tyrosine phosphorylated FRS2 provides four docking sites for Grb2 and two sites for interaction with the tyrosine phosphatase Shp2, which in turn also binds Grb2 [109]. Thus altogether, FRS2 leads to the direct and indirect recruitment of a large number of Grb2/Sos complexes, which makes it a major component in activation of the MAPK pathway. Further, FRS2 is also directly involved in signal downregulation. Phosphorylation on eight potential threonine residues by Erk1/2 is important for downregulation, since absence of these sites causes continuous FRS2 phosphorylation and cellular transformation [110, 111].

1.4.4.4 PLCγ

PLCγ binds to the site surrounding phosphorylated Y766 of FGFR1 via its SH2 domain. Substitution of this residue by phenylalanine abolishes PtdIns(4,5)P_2 hydrolysis without affecting the mitogenic activity of FGFR1 [112, 113].

1.4.4.5 c-Cbl

The E3 ubiquitin ligase c-Cbl is recruited via interaction of a proline-rich region with a Grb2 SH3 domain [114]. In conjunction with the E1 and E2 components of the ubiquitylation machinery, c-Cbl results in the ubiquitination of the FGFR, its subsequent internalisation and downregulation of the signalling pathway.

1.4.4.6 Sprouty

Sprouty (Spry) is an inhibitor of the Ras/Erk1/2 pathway in response to FGFR stimulation [115]. Phosphorylated Spry binds Grb2 and thereby prevents it from binding to FRS2 or Shp2. This induces a negative feedback mechanism on activation of Ras and subsequently Erk1/2 [116]. The overall mechanism of how Spry leads to downregulation of receptor signalling and Erk1/2 activation is not fully understood.

1.4.4.7 Shp2

Phosphorylated FRS2 contains two binding sites for the tyrosine phosphatase Shp2, which subsequently plays an important role in presenting additional binding sites for recruitment of Grb2/Sos complexes. In addition, its enzymatic activity is crucial for regulation of Erk1/2 activity, since inactive Shp2 is not able to sustain Erk1/2 phosphorylation in PC12 cells [109]. Shp2 dephosphorylates Spry and causes its dissociation from Grb2, which leads to a decrease in negative regulation via Spry [117].

1.4.4.8 Gab1

Gab1 binds to the C-terminal SH3 domain of Grb2 via a proline-rich region. It was demonstrated that formation of the FRS2/Grb2/Gab1 complex upon FGFR stimulation induced activation of PI3K as well as subsequent downstream proteins [26].

1.4.4.9 c-Src

To date there are several conflicting reports regarding the role of the Src kinase family in FGFR signalling. Although an interaction between Src and the FGFR1 has been reported [118], other reports failed to observe this direct association, and Src was found to be positively or negatively regulated by FGFR activation depending on the cell type used [119]. Recent reports have highlighted roles for Src in cell migration and shape changes via phosphorylation of cortactin [120] and suppression of Erk1/2 activity by resulting in Sprouty phosphorylation [121].

1.4.4.10 Other FGFR effectors

The adaptor protein Crk has been shown to bind to the FGFR1 at the site surrounding tyrosine 463. It interacts with various proteins including Cas, C3G and Shc, and has

been shown to play a role in the activation of the Erk1/2 and Jun kinases [122]. Furthermore, a recent study using tyrosine phosphoproteomics analysis by mass spectroscopy identified the insulin receptor substrate-4 (IRS-4) as a further protein involved in FGFR1 signalling [123]. The p120 Ras GTPase activating protein (RasGAP) has been shown to interact with and be phosphorylated by FGFRs in Drosophila and has been proposed to be involved in regulating the activation of Ras and subsequently the Erk1/2 pathway downstream of these receptors [124].

1.5 Specificity in receptor tyrosine kinase signalling

1.5.1 Multiple RTKs activate the same signalling pathways

All RTKs portray great similarities in the way in which they are activated and *trans*-autophosphorylate on tyrosine residues in their cytoplasmic regions. Moreover, they often induce the same cellular outcomes such as mitogenesis, apoptosis or differentiation. This leads to the question of whether different growth factors use the same or different intracellular signalling pathways to achieve these responses. Signalling proteins such as Grb2, Sos, Shc and Ras are involved in signalling from a variety of different RTKs and downstream targets like Erk1/2, PI3K or PLCγ can be activated by a plethora of different ligands (reviewed in [125]). Therefore the recruitment of a unique and specific protein complement to different RTKs cannot form the basis of specific and diverse signals.

Individual cell types usually express more than one type of RTK, which elicit very different cellular responses such as for example in PC12 cells, where EGF stimulation leads to proliferation, but NGF stimulation leads to differentiation [16]. Hence, although it is more efficient for cells to use the same signalling machinery, the knowledge that different receptors utilise common signalling proteins and activate the same overall pathways also presents a challenge. Quantitative and qualitative information is required to understand how specific signals are achieved and how crosstalk between signals from different receptors is avoided.

1.5.2 Avoidance of crosstalk and regulation of specificity

1.5.2.1 Interaction domain specificity

Many of the proteins involved in signal transduction from RTKs are made up of different combinations of a limited pool of small, modular domains. Each of these domains introduces specific functionality into a protein such as the ability to bind

phosphotyrosine (Src homology 2 (SH2), phosphotyrosine binding (PTB)), proline-rich regions (Src homology 3 (SH3), WW) or particular lipids such as phosphatidylinositols (pleckstrin homology (PH), PTB, FYVE). They are found in different combinations in many signalling proteins (Figure 1.5).

Signal transduction downstream of RTKs is often represented in the form of linear pathways, partly because of the ease of two-dimensional representation, but also because techniques such as immunoprecipitation, in vitro pulldown and yeast-two hybrid methods identify only high-affinity binding partners. However, for signal transduction to occur by linear protein-protein interactions, the affinity of a given protein for its binding partner, would have to be several orders of magnitude greater for the specific ligand over non-specific interactions [126]. If this was not the case, non-specific binding and activation of the incorrect signalling pathway could not be excluded and aberrant responses could be activated.

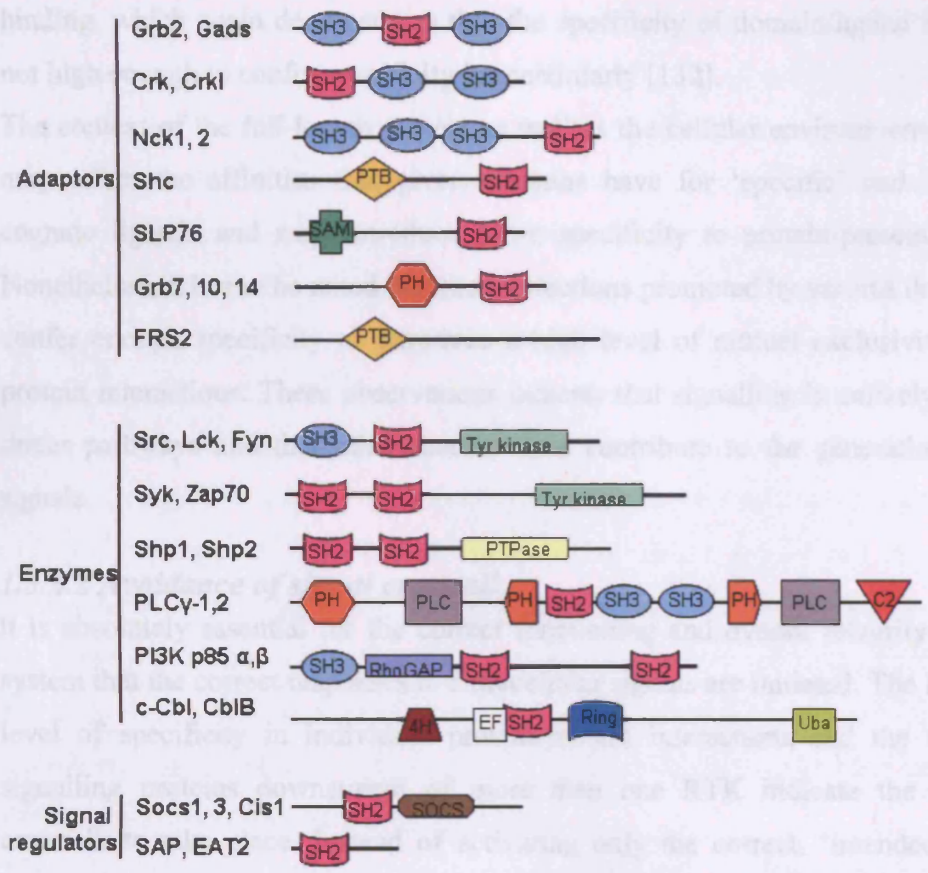


Figure 1.5: Diagrammatic representation of the types of proteins involved in signal transduction downstream of receptor tyrosine kinases Examples of SH2 domain containing proteins involved in signal transduction from RTKs are shown. Different combinations of various modular domains allow formation of proteins with different functionality. Abbreviations of the domains: SH2: Src homology 2, SH3: Src homology 3, PTB: phosphotyrosine binding, SAM: sterile alpha motif, PH: pleckstrin homology, Tyr kinase: tyrosine kinase, PTPase: tyrosine phosphatase, PLC: phospholipase, C2: calcium and phospholipid binding, RhoGAP: GTPase activating protein, Ring: Ring domain, EF: EF-hand, Uba: ubiquitin associated, SOCS: suppressor of cytokine signalling. Adapted from [127, 128].

Interactions of various SH2 domains with ligands have been analysed extensively in terms of binding affinities and specificities [129, 130]. Analysis of the dissociation constants for various phosphopeptides from the Src SH2 domain revealed that binding to a non-specific peptide sequence occurred with affinities of only an order of magnitude less than specific interaction with a pYEEI peptide [126, 131]. The affinities of different SH3 domains for various specific and non-specific ligands also varied by only two orders of magnitude [131]. Similarly, the Fyn SH3 domain can bind both Sos and the PI3K p85 subunit with similar affinities, so that discrimination of binding these two proteins cannot be achieved. Moreover, although some PH domains are able to differentiate between specific phosphatidylinositols, many portray fairly promiscuous

binding, which again demonstrates that the specificity of domain/ligand interactions is not high enough to confer specificity intracellularly [132].

The context of the full-length protein as well as the cellular environment/compartment may affect the affinities that given domains have for 'specific' and 'non-specific' cognate ligands and may introduce some specificity to protein-protein interactions. Nonetheless, it has to be noted that the interactions promoted by various domains do not confer enough specificity to introduce a high level of mutual exclusivity to protein-protein interactions. These observations indicate that signalling is unlikely to occur via linear pathways and that other factors must contribute to the generation of specific signals.

1.5.2.2 Avoidance of signal crosstalk

It is absolutely essential for the correct functioning and overall integrity of a cellular system that the correct responses to extracellular signals are initiated. The lack of a high level of specificity in individual protein-protein interactions and the use of many signalling proteins downstream of more than one RTK indicate the potential for crosstalk to take place. Instead of activating only the correct, 'intended' signal, the accidental activation of other pathways could occur, which would grossly affect the ultimate cellular response. One of the questions that arises, is how cells are able to avoid such crosstalk and how specific downstream outcomes such as differentiation, proliferation or apoptosis are achieved if the same signalling machinery is employed downstream of various RTKs.

1.5.3 Mechanisms regulating RTK signalling specificity

The need for regulation of RTK signalling beyond the recruitment of certain adaptor proteins to the activated receptor is apparent. There are several mechanisms by which specificity in RTK signalling is believed to be regulated, all of which are important in generating increased specificity in individual protein-protein interactions as well as regulating the duration and level of downstream signalling pathway activation. The following sections will provide an overview of the strategies employed to induce specific biological responses.

1.5.3.1 Multiprotein complex formation

One of the major regulators of specificity in RTK signalling is the formation of multiprotein complexes. Their assembly allows more precise spatio-temporal control over the signal transmitted from the receptor to the inside of the cell than simple linear

pathways. The combinatorial recruitment of proteins into complexes specific to the respective receptor via their various modular domains provides a means by which specificity can be achieved. Differences in the types of proteins recruited and in their arrangement in a complex as well as in the duration for which such assemblies exist allow the unique and specific activation of signalling pathways downstream of different receptors. Additionally, because different proteins donate various functionalities to a multiprotein complex, regulated activation of the desired complement of signalling pathways can be achieved.

Moreover, the formation of complexes introduces more specificity into individual protein-protein interactions by allowing formation of multivalent interactions. The binding affinities of individual domains for its target ligand can be increased by cooperativity in the context of the whole protein [133, 134]. Specificity is introduced in the form of a requirement for the complete and precise protein complement to be recruited in order to allow unique signals to be generated (Figure 1.6). The formation of complexes affects factors such as affinity and availability of binding partners, which allows more specific signals to be generated.

The formation of multiprotein complexes also provides flexibility to the signal created. The composition of the complex and the stoichiometry of proteins can change with time, thereby providing tight system regulation throughout prolonged receptor engagement. In linear pathways such fine-tuned control would not be possible since proteins would either interact or not. The complexes that assemble upon activation of for example the T-cell receptor are highly dynamic and rapid changes in complex composition occur, thereby providing a high level of spatial and temporal control [135]. Additionally, the longevity of signals can also be more tightly controlled because proteins in complexes may for example be shielded from dephosphorylation. On the other hand the controlled recruitment of negative regulators such as protein phosphatases and ubiquitin ligases to a complex is also important in the creation of specificity as it allows regulated signal downregulation.

Altogether, the spatio-temporal regulation of recruitment of proteins into these complexes is an extremely important aspect in generation of the unique and specific signals generated by various RTKs.

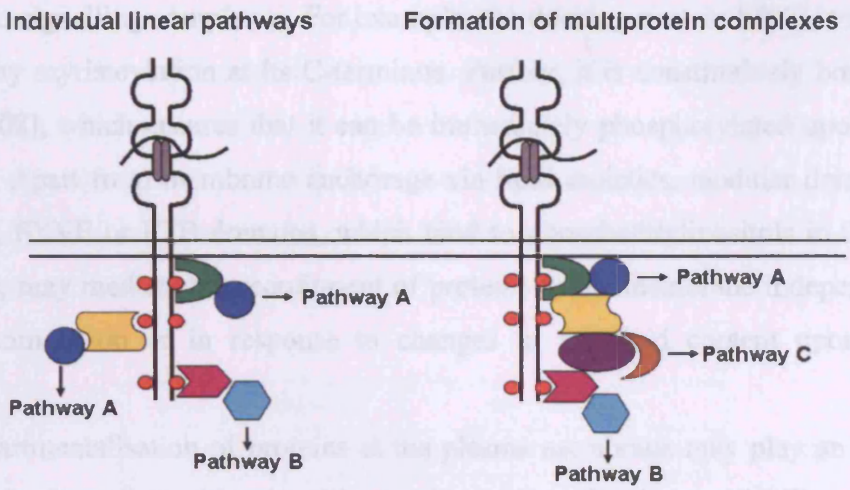


Figure 1.6: Signal transduction from RTKs via linear pathways or formation of multiprotein complexes Linear representation of cell signalling involves each protein binding to a maximum of two binding partners and activation of a limited number of pathways. Individual protein-protein interactions would have to be highly specific to allow activation of the correct pathways and to avoid signal crosstalk. Formation of a multiprotein complex allows recruitment of additional proteins via multivalent interactions with various binding partners, which leads to the activation of additional pathways specific to the receptor activated and complex formed. Recruitment of a precise protein complement into multimolecular complexes downstream of different RTKs introduces specificity and allows unique activation of signalling pathways.

1.5.3.2 Use of scaffold proteins

Scaffold proteins anchor components of signalling pathways to certain cellular locations or prevent activation unless the right conditions are present. Thereby scaffold proteins can sequester signalling components and prevent activation by closely related pathways (reviewed in [136]). Scaffolding proteins such as kinase suppressor of Ras-1 (KSR-1), β-arrestin and paxillin are important for the specific activation of the Ras/Raf/MEK/Erk1/2 pathway [137]. Such proteins are also important in regulating the avoidance of crosstalk with other pathways and target components to specific locations, provide specific signal inhibition and affect assembly and turnover of different components. Scaffold proteins thereby add additional specificity to the signal transduced and play an important role in avoiding crosstalk between different pathways/signals.

1.5.3.3 Compartmentalisation of signalling

Many of the proteins involved in cell signalling are not found ubiquitously in the cytoplasm, waiting for a given RTK to be activated. Instead, many proteins are already targeted to the membrane or kept in locations from which they can be more readily

recruited to signalling complexes. For example, the docking protein FRS2 is membrane anchored by myristoylation at its C-terminus. Further, it is constitutively bound to the FGFR1 [108], which ensures that it can be immediately phosphorylated upon receptor activation. Apart from membrane anchorage via lipid moieties, modular domains such as the PH, FYVE or PTB domains, which bind to phosphatidylinositols in the plasma membrane, may mediate the recruitment of proteins to the membrane independently of receptor stimulation or in response to changes in the lipid content upon receptor activation.

The compartmentalisation of proteins at the plasma membrane may play an important role in activation of specific signalling pathways by affecting availability of various downstream signalling proteins. Lipid rafts have been proposed as regions that allow pre-sequestration of various proteins involved in signal transduction (reviewed in [138]). The recruitment of proteins to the membrane increases their effective concentration, which may increase the apparent affinity for binding partners up to 1000-fold [139]. Recruitment of proteins to the same cellular compartment further affects the longevity of complexes as a result of the increase in local concentration [126].

The relocation of proteins as a result of activation by RTKs is another aspect of cellular compartmentalisation that is also important in the generation of specific and diverse signals. For example, the translocation of Erk1/2 to the nucleus upon exposure of PC12 cells to NGF but not to EGF [140] marks a difference between sustained and transient activation and is therefore an important regulator of signal specificity.

1.5.3.4 Regulation of signal duration and amplitude

In PC12 cells, prolonged Erk1/2 signalling (following NGF or FGF stimulation) leads to differentiation, whereas transient activation of this kinase (in response to EGF or insulin) causes proliferation [16]. Other models have also shown that the difference between transient and prolonged activation of the Erk1/2 pathway is important in regulating signal specificity (Figure 1.7). For example, quiescent fibroblasts enter S phase and begin to proliferate in response to prolonged but not transient Erk1/2 activation [141, 142]. The amplitude of a signal is also important in determining the ultimate cellular response. In the case of PC12 cells, overexpression of the EGFR or the IR was also able to induce differentiation [140, 143]. Similarly, the survival of carcinoma cells is linked to a strong Erk1/2 activity, whereas a lower level of Erk1/2 activation leads to cell death by apoptosis [144].

The quantitative regulation of signals in terms of duration and magnitude is therefore an important factor in regulating cellular responses (Figure 1.7). The correct balance between stimulatory and inhibitory signals is required to regulate both strength and duration of a signal and lead to the correct cellular response. This provides an interesting basis for investigation of the effects of mutations in various signalling components on signal duration and magnitude.

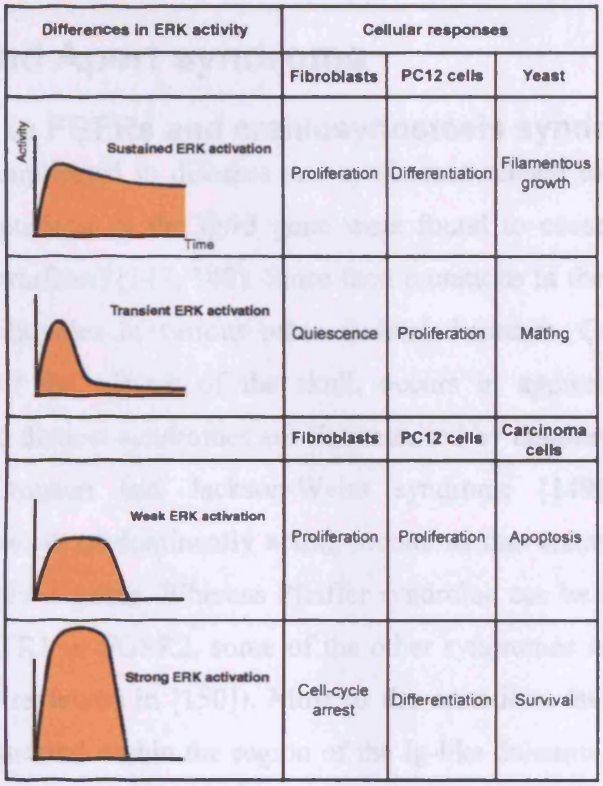


Figure 1.7: Schematic representation of the importance of quantitative regulation of Erk1/2 signalling for the generation of signalling specificity Specific celluar responses can be mediated by quantitative differences in the activation of signalling pathways such as the Erk1/2 pathway. Adapted from [145].

1.5.3.5 Specificity as a result of cellular context

Different expression patterns of receptors and ligands are important in contributing to cell-specific activation of signalling pathways and subsequent cellular responses. For example, PC12 cells do not express any PDGFR, and thus are normally unresponsive to PDGF. However, when the gene encoding the PDGFRβ is expressed in PC12 cells, PDGFβ is able to induce differentiation [146]. In addition, cells may also express a certain protein complement, which directs the specific activation of certain pathways but does not allow other pathways to be activated. FGF stimulation of NIH 3T3

fibroblasts leads to proliferation, but FGF stimulation of PC12 cells leads to their differentiation into neurons [16]. These differences are most easily explained by cell-type specific expression of effector proteins that are able to mediate different up- or downregulation of transcription factors and gene expression in response to RTK activation, which provides a further basis for the achievement of specificity in RTK signalling.

1.6 FGFR2 and Apert syndrome

1.6.1 Mutations in FGFRs and craniosynostosis syndromes

FGFRs were first implicated in diseases and syndromes related to bone development when activating mutations in the *fgfr3* gene were found to cause achondroplasia (a common form of dwarfism) [147, 148]. Since then mutations in the *fgfr1-3* genes have been implicated with roles in various other skeletal disorders. Craniosynostosis, the premature fusion of the sutures of the skull, occurs in approximately 1 in 2500 newborns. Over 100 distinct syndromes are characterised by craniosynostosis, including Apert, Pfeiffer, Crouzon and Jackson-Weiss syndrome [149]. Craniosynostosis syndromes are the result of dominantly acting mutations that create a gain-of-function phenotype in the *fgfr1-3* genes. Whereas Pfeiffer syndrome can be caused by a number of mutations in FGFR1 or FGFR2, some of the other syndromes are caused by highly specific mutations (reviewed in [150]). Most of the mutations that affect craniofacial development are clustered within the region of the Ig-like domains D2 and D3 and the linker region between them.

1.6.2 The molecular basis of Apert syndrome

Apert syndrome was first described in 1906 [151]. Besides craniosynostosis, Apert syndrome is characterised by severe syndactyly, abnormalities of the skin, skeleton, brain and other internal organs [152]. Syndromes such as Crouzon syndrome are caused by the constitutive activation of the FGFR2, which results in a gain-of-function phenotype. This phenomenon occurs as a result of creation of unpaired cysteine residues that form intermolecular disulphide bridges, which cause ligand-independent receptor dimerisation and activation [153-155]. In contrast, Apert syndrome is primarily caused by one of two mutations in the linker region between the D2 and D3 Ig-like domains in the extracellular region of FGFR2 (Figure 1.8). The S252W and P253R mutations occur in approximately 65% and 35% of all cases respectively [152, 156]. Other mutations

can also cause Apert syndrome, but they make up a very small proportion of all cases. These mutations include the S252F [150] substitution, a de novo Alu-element insertion that leads to altered expression of the two FGFR2 splice variants [157], and double mutations such as S252L/A315S, which cause syndactyly only when both mutations are present [158].

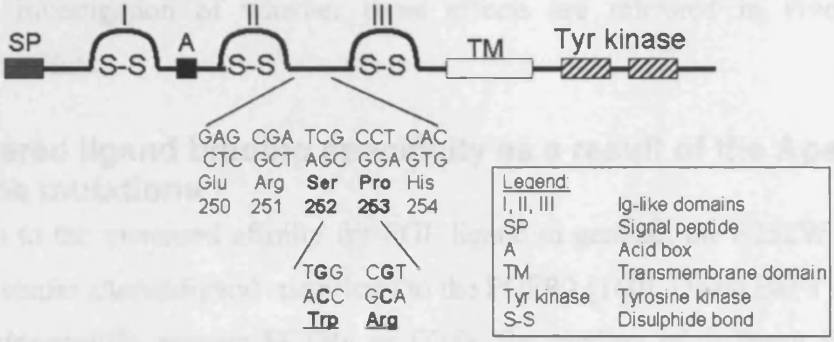


Figure 1.8: Location of the S252W and P253R Apert syndrome mutations Both the S252W and the P253R mutations are located in the linker region between the Ig-like domains II and III of the FGFR2. This region contributes to ligand binding and recognition. The single base pair substitutions leading to amino acid substitutions S252W and P253R are indicated. Adapted from [159, 160].

1.6.3 Increased binding affinity of Apert syndrome FGFR2 for FGF

Despite resulting in a similar phenotype as the Crouzon mutations, the Apert syndrome mutations do not cause ligand-independent dimerisation [161]. In contrast, the Apert syndrome mutations result in increased affinity of the mutant receptors for FGF ligand compared to the normal FGFR2 [7, 161]. Various approaches have revealed slightly different results regarding the extent to which the Apert syndrome mutations increase affinity for FGF ligand, which FGFs portray this effect and whether only the dissociation constant (k_{off}) or both the association and dissociation constants (k_{on} and k_{off}) are affected. While one group expressed and purified the soluble extracellular domain of FGFR2(IIIc) (Ig-like domains D1-D3) in HEK 293 cells [161], another group used residues 147-366 of the FGFR2IIIc expressed in *E.coli* that had to be refolded from inclusion bodies [7]. Whereas the construct expressed in mammalian cells would be fully glycosylated, the bacterially expressed protein lacks this posttranslational modification, which would affect the FGFR-FGF interactions taking place [162]. Additionally, the bacterially expressed protein that was refolded from inclusion bodies may not possess the correct disulphide bridges, and is lacking the D1 Ig-like domain, which has been shown to be an important regulator of ligand binding [77]. However,

despite slight discrepancies, both studies reported an overall increased affinity of the Apert syndrome mutant receptors and that this effect was generally more pronounced in the case of the S252W mutation.

Despite extensive studies of the effect of the Apert syndrome mutations on ligand binding in vitro, and a report outlining the increased mitogenic response of BaF3 cells expressing the S252W mutant receptor compared to the normal FGFR2 to some FGFs [160], no investigation of whether these effects are mirrored in vivo has been undertaken to date.

1.6.4 Altered ligand binding specificity as a result of the Apert syndrome mutations

In addition to the increased affinity for FGF ligand in general, the S252W and P253R mutations confer altered ligand specificity to the FGFR2 [160]. Using BaF3 cells, which do not endogenously express FGFRs or FGFs, the binding of different FGFs to the receptor was assessed by means of a mitogenic assay. The presence of the S252W mutation allowed activation of the FGFR2(IIIc) by FGF7 and FGF10, which are normally high affinity ligands for FGFR2(IIIb) and do not activate the FGFR2(IIIc) isoform. Similarly, cells expressing the S252W FGFR2(IIIb) showed a robust mitogenic response upon stimulation with FGF2, 6 and 9, all of which did not elicit any response in cells expressing the wild type FGFR2(IIIb).

1.6.5 Structural basis for the increased affinity and altered specificity in the presence of the Apert syndrome mutations

The crystal structure of the FGFR2(IIIc) extracellular domain (residues 147-366) does not significantly change in the presence of the S252W or P253R mutations [163]. However, additional contacts between the mutant receptors and the ligand are present (Figure 1.9). In case of the S252W mutant, Trp252 stabilises the formation of a hydrophobic patch (involving Trp252, Tyr281 and Ile257) that can be engaged by Phe21 of FGF2 (refer to Figure 1.9). Stabilisation of Tyr281 in a single conformation allows formation of a new hydrogen bond with FGF2 Pro22 and of oxygen-aromatic interactions with the backbone oxygen of Gly24 of FGF2. In the presence of the P253R mutation, the arginine residue introduced forms three additional hydrogen bonds with Leu107, Glu108 and Asn111 of FGF2 (Figure 1.9). In both cases such additional contacts would be expected to result in increased affinity for the ligand. Although these findings support the increased binding of some FGFs to the S252W receptor, they cannot really explain the increased affinity of the receptor for ligands such as FGF10,

which do not possess a hydrophobic residue in the position corresponding to Phe21 in FGF2. Thus other mechanisms that rely on flexibility of the system that cannot be represented in a crystallographic model must be involved in order to accommodate the increased affinity of mutant receptors for such FGF ligands [163].

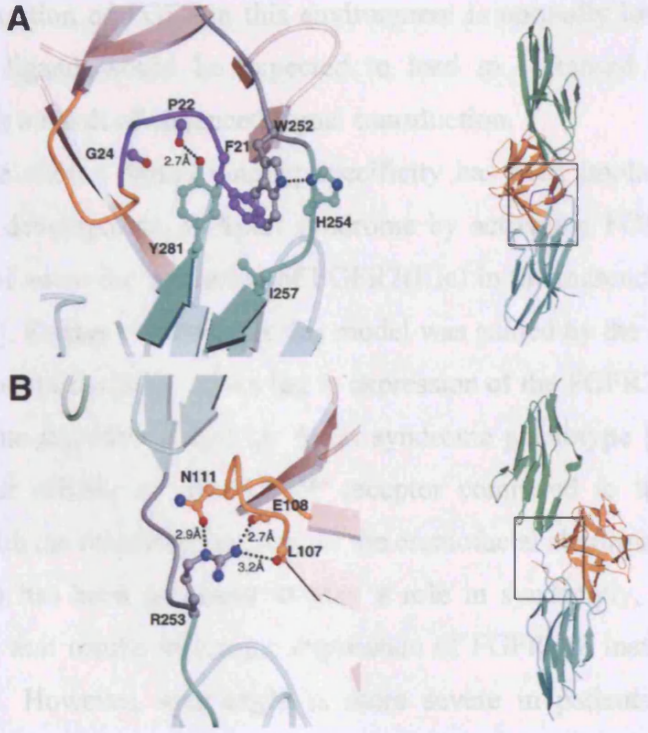


Figure 1.9: Additional contacts between FGF2 and the S252W (A) and P253R (B) Apert mutant receptors Additional contacts are made with FGF2 in the case of both substitution mutations, which affect the affinity of the FGFR2 for FGF ligand. The S252W and P253R receptors are presented in different orientations as indicated (right hand panel). Refer to section 1.6.5 for an explanation of the additional contacts made. D2 and D3 of FGFR2 are shown in green and cyan respectively, the D2-D3 linker is shown in grey. FGF2 is shown in orange. Oxygen atoms are red, nitrogen atoms blue, and carbon atoms are coloured in the same shade as the molecule they belong to. Hydrogen bonds are represented by dotted lines. Extracted from [163].

One of the major limitations of assessing the differences in ligand binding by the mutant receptors using bacterially expressed proteins and crystallography is that regulation of ligand binding by posttranslational modifications such as glycosylation and the addition of heparan sulphate are not taken into consideration. The system also lacks flexibility, which might be important in the physiological context, which indicates the need to further investigate these effects on the cellular level. Thus the effects of the mutations observed in vitro may not necessarily hold true to the same extent in vivo, where additional factors may play a role in terms of mediating the receptor:ligand interaction.

1.6.6 Models for the manifestation of Apert syndrome phenotypes

Based on early studies reporting increased affinity of the FGFR2(IIIc) mutant receptor for ligand it was proposed that this could be sufficient to cause craniosynostosis. Since FGFR2(IIIc) and FGF ligand are only expressed in a restricted region of the coronal suture this area would be the most obvious target for the mutation to take effect [161]. Since the concentration of FGF2 in this environment is normally low, the increase in affinity for this ligand would be expected to lead to increased differentiation of osteogenic cells as a result of enhanced signal transduction.

More recently the altered ligand binding specificity has been implicated as the main factor leading to development of Apert syndrome by activating FGFR2 in the wrong context because of autocrine activation of FGFR2(IIIc) in the mesenchyme by FGF7 or FGF10 [5, 81, 99]. Further evidence for this model was gained by the description of two de novo Alu-element insertions, which led to expression of the FGFR2(IIIb) splice form in the mesenchyme and also caused an Apert syndrome phenotype [157]. The greater increase in ligand affinity of the S252W receptor compared to the P253R mutant correlates well with the respective severity of the craniofacial abnormalities.

The FGFR2(IIIb) has been proposed to play a role in syndactyly, because the Alu-element insertion that results in ectopic expression of FGFRIIIb instead of IIIc causes syndactyly [157]. However, syndactyly is more severe in patients with the P253R mutation, whereas the affinity increase for FGFs was greater in the case of the S252W mutation [7] This may indicate that the FGFR2(IIIb) is not solely responsible for leading to the development of syndactyly and that other factors than the increase in affinity of the receptors for ligand may play a role in causing the Apert syndrome phenotypes observed.

1.6.7 Effects of Apert syndrome mutations on the cellular basis

1.6.7.1 Effects on osteoblast differentiation

Several studies have reported an increase in osteoblast apoptosis when the S252W FGFR2 is expressed [164, 165]. An increase in premature apoptosis of differentiated osteoblasts and osteocytes with the S252W mutation was also observed in vivo [166]. Mansukhani *et al.* further described lower levels of alkaline phosphatase expression, a protein that is indicative of differentiation, as well as decreased mineralisation in cells expressing the S252W receptor compared to the wild type FGFR2 [164]. In contrast, other studies reported an increase in differentiation in cells expressing the Apert mutant FGFR2IIIc compared to control cells as well as a reduced apopotic rate in the presence

of the S252W mutation [167, 168]. The conflicting results reported may be due to the different sources from which cells were derived. The effects observed on osteoblast proliferation and/or differentiation are greatly dependent on the maturation stage of the cells [165]. Indeed, opposite effects of FGF on calvaria cell differentiation, apoptosis or proliferation were observed in mature and immature osteoblasts. Nonetheless, this conflicting evidence makes comparison and analysis difficult and prevents a clear picture of the intracellular effects of Apert syndrome from being formed.

1.6.7.2 Effects on intracellular signalling

Despite the investigations trying to elucidate the cellular basis of Apert syndrome, few reports describe the effects of the mutations on the actual signalling pathways downstream of the FGFR2. In cells expressing the S252W FGFR2, the genes encoding PKC, interleukin 1α and RhoA are upregulated and phosphorylation of PLCγ and PKC is increased [169, 170]. Additionally, the levels of Src, Fyn and Lyn are downregulated by Cbl-mediated targeting for proteasomal degradation in cells expressing the S252W [171]. Several reports have also indicated downregulation of the Apert mutant FGFR2 in cells expressing the Apert mutant receptors [166, 171, 172]. In addition, increased levels of E-cadherin and N-cadherin have been described in S252W-expressing cells, which are important in cell adhesion and may play a role in intracellular signalling [169]. The S252W FGFR2 did not lead to enhanced Erk1/2 activation in response to FGF1 stimulation in transfected osteoblasts [164]. However, when using different FGFs such as FGF9 mitogenesis was greatly upregulated in cells expressing the S252W FGFR2, which indicates that perhaps the Erk1/2 pathway and others regulating mitogenesis are indeed upregulated in cells expressing the Apert mutant receptors [160]. The lack of studies focusing on the intracellular signalling downstream of the Apert mutant receptors is apparent. Although the Apert mutations have been classed as gain-of-function mutations [173], no studies have truly investigated the effects of the mutations on early signalling events initiated by the FGFR2. A detailed study into the effects of both the S252W and the P253R on intracellular signalling is required to understand the cellular mechanisms that contribute to the manifestation of Apert syndrome.

1.6.7.3 Effects of the Apert syndrome mutations in non-osteoblastic cells

In addition to affecting differentiation and apoptosis in osteoblasts, the Apert mutations have been shown to result in positive selection in sperm cells [173]. The fact that mutant receptors portrayed this gain-of-function effect in mature spermatogonia prompted an

investigation into whether they were involved in tumour formation [174]. However, out of 77 tumours investigated, none showed expression of the Apert mutant FGFR2. Thus the gain-of-function effect is not necessarily transferable to all kinds of systems and plays specific roles in certain cellular contexts. This means that the Apert mutations confer very specific cellular effects, which only manifest themselves in cells in which FGFR2 signalling plays an important role. More detailed studies of intracellular signalling downstream of the mutant FGFR2 would be required to elucidate how different cellular phenotypes arise.

1.7 The signalling adaptor protein Shc

1.7.1 Shc domain composition and structure

1.7.1.1 Shc isoforms

Three different forms of the Shc adaptor are expressed in mammals: ShcA, ShcB (also known as Sck) and ShcC, of which ShcB and ShcC are predominantly expressed in the brain ([175-177]. ShcA (most commonly and from hereon referred to as Shc) is ubiquitously expressed and exists as three isoforms, namely p66Shc, p52Shc and p46Shc, where the numerical values reflect their relative molecular weight. These are encoded by two different transcripts generated from the same gene by use of alternative 5' exons [178]. The p52 and the p46 isoforms are produced from the same transcript by use of alternative start sites.

1.7.1.2 Domain composition

All three Shc isoforms contain a collagen homology domain (CH1, a region rich in proline and glycine residues but with no resemblance to the collagen fold) flanked by an SH2 domain on the C-terminus and a PTB domain on the N-terminus. The p66 isoform contains an additional CH2 domain at the very N-terminus [175].

1.7.1.3 Shc structure

The structure of full-length Shc has not been solved. However, the structure of the isolated SH2 domain has been determined [179, 180], and has been shown to essentially portray the same fold as other SH2 domains with some additional loops. The structure of the PTB domain has also been solved, which revealed a fold similar to that of the pleckstrin homology (PH) domain but with the ability to bind phosphotyrosine [181]. The PTB domain on its own is fairly unstructured, and only folds upon ligand binding

[182]. Despite its name, the CH1 domain does not form a collagen-like fold, but the exact structure of this domain is unknown.

1.7.2 Role of Shc in RTK signalling

Shc was one of the first signalling proteins studied in detail and to date has been implicated in a large number of cellular functions ranging from differentiation to regulation of mitogenesis, cell adhesion and migration. When first discovered, Shc was reported to bind to and be phosphorylated by the EGFR [175]. Since this discovery, Shc has been implicated in signal transduction from an enormous variety of tyrosine kinase receptors, including the insulin receptor, FGFR, TrkA, PDGFR, hepatocyte growth factor receptor and others [183].

Shc binds to phosphotyrosine motifs on different tyrosine kinase receptors following ligand activation via either its PTB or SH2 domain or a combination of both [184]. In addition, the similarity of the PTB domain fold to that of the PH domain allows it to interact with phosphatidylinositols in the plasma membrane and can be recruited to the sites of activated receptors in this way [185]. Once recruited to the vicinity of activated RTKs, Shc is phosphorylated on two major sites within the CH1 domain, tyrosine 317 and the dual site consisting of tyrosines 239 and 240 [72]. Both form binding sites for Grb2, via which Sos can be recruited to Shc and hence the respective activated RTK. This leads to activation of the Ras/Raf/MEK/Erk1/2 pathway. Despite its ability to interact with various other proteins, the main role for Shc in RTK signalling seems to lie in providing additional Grb2 binding sites and the assembly of larger signalling complexes downstream of tyrosine kinase receptors to regulate Erk1/2 activation.

1.7.3 Role of individual Shc domains

1.7.3.1 The phosphotyrosine binding (PTB) domain

The involvement of the Shc PTB domain in cell signalling was first described by Blaikie *et al.* [184]. It has since been shown to be the major component of Shc mediating interaction with various growth factor receptors. The minimal motif required for Shc PTB domain interaction is the sequence NPXpY and the authors proposed that the motif "hydrophobic residue-(D/E)-N-X-X-pY-(W/F)" as found in the Trk and ErbB receptors allows the highest affinity interaction with the Shc PTB domain [186].

The Shc PTB domain has been shown to interact with the EGFR and the insulin receptor [50], HER2/neu and TrkA [184]. The NPXY motif around Y490 in the TrkA receptor functions as a binding site for both FRS2 and Shc [4], and competition between

these two proteins exists for binding at this site. In contrast, the FRS2 PTB domain binding site on the FGFR1 does not need to be phosphorylated for FRS2 to bind and does not contain the NPXY motif required for interaction with the Shc PTB domain. Thus a competition model for this site is unlikely. Different modes of Shc interaction with various receptors may regulate specific involvement of this adaptor protein in different pathways and complexes.

Since the fold of the PTB domain is similar to that of the PH domain it is able to bind to acidic phospholipids (such as phosphatidylinositols) [181]. Phospholipid and phosphotyrosine binding to the PTB domain are independent of each other and both properties are required for Shc to be able to localise to the membrane and carry out its functions in RTK signalling [185]. The ability of Shc to be recruited to the membrane via phospholipid binding could also explain how it retains the ability to be phosphorylated and activate the Erk1/2 signalling pathway in cells that express only a truncated EGFR or an FGFR1 lacking all major autophosphorylation sites [103, 187, 188]. Altogether the PTB domain is important for localisation of Shc to the membrane and binding to the receptor, which can then lead to its phosphorylation.

1.7.3.2 The Src homology 2 (SH2) domain

The structure of the Shc SH2 domain bound to peptide has been solved [179, 180]. Although the overall fold was similar to that of other SH2 domains, analysis by nuclear magnetic resonance (NMR) revealed that the interaction with the phosphotyrosine peptide was slightly different. The phosphotyrosine moiety bound the SH2 domain in a similar fashion to that observed for other SH2-peptide complexes. In contrast the pY+3 residue of the peptide did not make significant contact with the protein [180].

The SH2 domain has been shown to interact with the EGFR, albeit at slightly lower affinity than the PTB domain [49]. Other reports have also implemented the SH2 domain as an important factor in EGFR signalling [50, 72, 189]. Further, the SH2 domain has been shown to bind the T-cell receptor ζ-chain, which indicates that it plays a role in Shc recruitment to activated cell surface receptors in addition to the PTB domain [190, 191].

Although generally physiological binding partners for the Shc SH2 domain are not very well characterised, the SH2 domain has been shown to also mediate functions other than recruitment of Shc to tyrosine kinase receptors. For example, it can bind CEACAM (a 120kDa phosphoprotein involved in regulating mitogenic activity downstream of the

insulin receptor [192]), cadherin and integrins (which are involved in cell adhesion and cell-cell interactions [193-195]), the E3 ubiquitin ligase c-Cbl [196] and Gab2 [197].

1.7.3.3 The collagen homology 1 (CH1) domain

The main role of the CH1 domain is to provide the two phosphorylatable motifs for Grb2 binding, Y317 and Y239/Y240. For a long time the Y317 site was deemed the only and most important site of Grb2 binding, but the Y239/Y240 site plays an important role in mediating PC12 cell differentiation in response to TrkA stimulation by NGF [72]. It was also shown that both tyrosine residues in the Y239/Y240 motif were necessary for functionality, although Y239 is the only residue that is part of a pYXNX consensus sequence for Grb2 binding [198].

Although the main role of the CH1 domain is recruitment of the Grb2/Sos complex, and both the Y317 and the Y239/Y240 sites are important for this function, it has also been shown that the individual sites carry out pleiotropic and nonredundant roles in the regulation of JNK and p38 MAPK activation, cell death and c-*myc* transcription in response to T-cell receptor activation [199]. Further, a CH1-domain deletion mutant can suppress ErbB2-induced transformation by a mechanism separate from Erk1/2 activation [200]. In addition, analysis of the amino acid sequence of the CH1 domain reveals the presence of potential sites for SH3 domain binding, which may play a previously unidentified role in Shc interaction with various binding partners.

1.8 Use of fluorescent proteins to study signal transduction

1.8.1 Ways to visualise proteins and signalling events intracellularly

1.8.1.1 Antibodies, protein tagging and fluorescent proteins

To analyse the spatio-temporal changes that different signalling proteins undergo upon stimulation of cells with a given growth factor, a method for visualising their localisation, and changes thereof, is essential. Methods such as membrane fractionation are useful to analyse protein movement between different cellular or membraneous compartments. However, more information can be gained from studies visualising such changes intracellularly.

Antibodies against specific cellular proteins that are detected using a secondary antibody tagged with an organic dye, a phycobiliprotein or quantum dots or are directly labelled with a fluorophore such as Cy3 or Cy5 can be used to analyse protein

localisation intracellularly [201]. Some of the problems of this technique are the requirement of highly specific antibodies or the need to tag the target protein with an epitope tag, the size of the immuno-complex that might interfere with protein recognition as well as its restricted use only in permeabilised cells. Enzymatic tags such as peroxidase, beta-galactosidase, luciferase or beta-lactamase can also be used to study the localisation of proteins intracellularly [202, 203].

The use of fluorescent protein (FP) tags such as the 27kDa green fluorescent protein has made visualisation of proteins easier. However, their use has disadvantages such as exogenous expression in a normal background system, the requirement of overexpression and the size of FPs that may interfere with protein localisation or function [201, 203]. The use of fluorescent proteins allows the investigation of direct protein-protein interactions using techniques based on energy transfer between individual fluorophores (refer to section 1.8.4). This makes them a preferred choice to study spatial and temporal changes in recruitment of various adaptor proteins to RTKs and their multiprotein complexes despite the caveats described.

Systems such as the use of a tetracysteine-biarsenical motif provide alternatives to fluorescent proteins. This 12 amino acid tag containing four cysteines is fused to target proteins and is able to bind biarsenical dyes such as the green 'FlAsH' and red 'ReAsH' with picomolar affinity which allows intracellular visualisation [204]. However, exogenous expression is still required, toxicity to endogenous processes cannot be excluded, higher fluorescent background occur and the system does not allow the use of two different colours in the same cellular compartment (reviewed in [201]).

1.8.1.2 Conventional microscopy compared to confocal microscopic imaging

Imaging of protein localisation can be carried out using conventional microscopy techniques or confocal microscopic imaging. Although conventional microscopy is straightforward to use it does have some drawbacks. For example, due to the uneven shape of cells, protein localisation in membrane invaginations or caveolae may be misinterpreted due to the lack of three-dimensional information [203].

The use of confocal microscopy allows an increase in resolution by a factor of up to 1.4 [203]. Moreover, the great advantage of confocal microscopy lies in being able to view optical sections through the sample and therefore gaining more information about the three-dimensional distribution of proteins. Optical sectioning also avoids out-of-focus signals from outside the focal plane.

1.8.2 Fluorescent proteins

The green fluorescent protein (GFP, Figure 1.10A) was first cloned from *Aequorea victoria* in 1994 [205], which was shortly followed by its use to measure gene expression and to monitor protein trafficking intracellularly [206, 207]. However, the early fluorescent proteins possessed some disadvantages such as reduced photostability, slow folding intracellularly and formation of multimers. More recently, various modified versions of GFP have become available which are more photostable, fold easily at 37°C, are mostly monomeric and can be excited at different wavelengths ([208] and reviewed in [209]). Most importantly, through several mutations, a number of fluorescent proteins were created that display very different excitation/emission spectra, such as yellow, orange and cyan varieties (Figure 1.10B). This allows the use of more than one FP to be employed in the same system without spectral overlap, which is important for studies using co-localisation or FRET (see below).

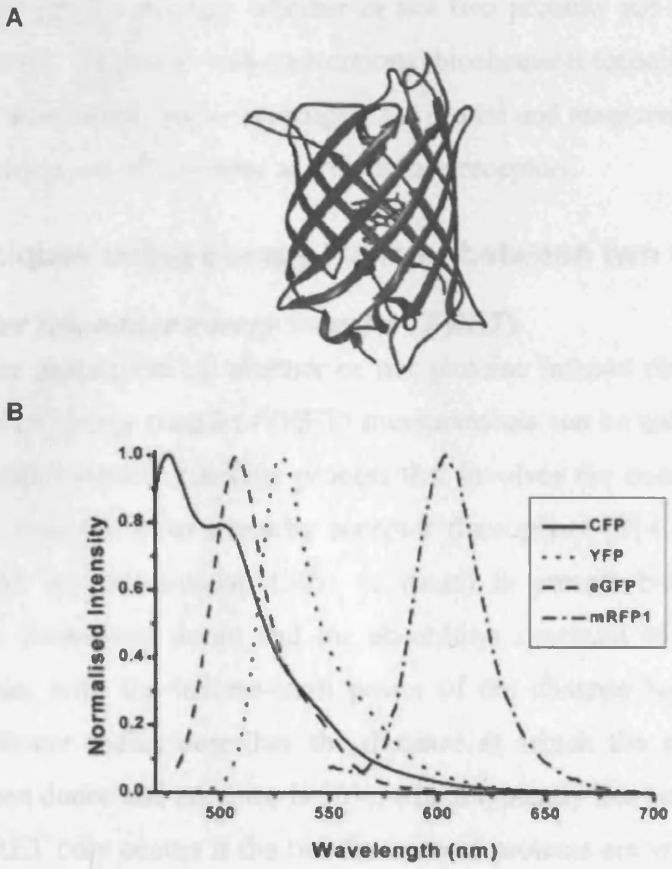


Figure 1.10: Structure of GFP and emission spectra for commonly used fluorescent proteins The structure of GFP is shown in form of a ribbon diagram. The tripeptide forming the fluorophore is indicated (A). Adapted from [210]. The emission spectra of four commonly used fluorescent proteins variants (cyan (*CFP*), yellow (*YFP*), enhanced green (*EGFP*), and monomeric red (*mRFP1*) fluorescent protein) are shown as indicated. (B) Adapted from [211].

1.8.3 Different techniques using fluorescent proteins

1.8.3.1 Single protein labelling

The discovery of fluorescent proteins created an entirely new avenue for studying intracellular signalling and protein trafficking in response to various stimuli. On the simplest level, a single protein can be tagged with a FP and its cellular localisation can be assessed microscopically. This approach has been used to study the trafficking and functions of proteins such as the EGFR and TrkA and has revealed new insight into the dynamics of these receptors upon ligand stimulation [13, 212].

1.8.3.2 Protein co-localisation

Although the use of single FP-tagged molecules is useful to obtain data on the intracellular dynamics of proteins, more information can be gained from the use of different FPs fused to two proteins that are, for example, part of the same signalling cascade (reviewed in [213]). Co-localisation alone cannot indicate direct protein-protein interactions and simply portrays whether or not two proteins are in the same cellular location. However, combined with conventional biochemical techniques, co-localisation studies can be a powerful tool to investigate the spatial and temporal changes in protein-protein interactions and recruitment to cell surface receptors.

1.8.4 Techniques using energy transfer between two fluorophores

1.8.4.1 Förster resonance energy transfer (FRET)

For an accurate assessment of whether or not proteins interact directly intracellularly Förster resonance energy transfer (FRET) measurements can be used. FRET refers to a nonradiative, dipole-dipole coupling process that involves the energy transfer from an excited donor fluorophore to a nearby acceptor fluorophore [214, 215]. FRET occurs when sufficient spectral overlap (30% or more) is present between the emission spectrum of a fluorescent donor and the absorption spectrum of an acceptor. FRET efficiency varies with the inverse-sixth power of the distance between acceptor and donor. The Förster radius describes the distance at which the efficiency of energy transfer between donor and acceptor is 50%, which typically lies between 1-10nm [211, 216]. Thus FRET only occurs if the two fluorescent proteins are within this distance of each other. If the donor is excited, this leads to emission at a particular wavelength within the excitation range of the acceptor. Thus if fluorescent donor and acceptor are in close enough proximity and in favourable orientation to each other, the transfer of energy leads to emission from the acceptor which would not be observed in the absence

of FRET. By detecting emission at the wavelength of the acceptor emission spectrum FRET can be measured.

Since FRET only occurs over a very short distance, it is an accurate technique for measuring direct protein-protein interactions in vivo and effectively allows these interactions to be assessed at nanometer resolution. FRET depends on favourable orientation of the donor and acceptor molecules (i.e. on the way in which the respective attached protein interact) as well as on the stoichiometry of donor and acceptor molecules. Nonetheless, it is far superior over simple co-localisation techniques. Whereas co-localisation studies are only able to indicate whether two proteins are located in the same cellular region or compartment, FRET allows the assessment of complex assembly and disassembly with time inside the cell.

CFP and YFP are a widely used pair of fluorescent proteins for FRET experiments, although various problems are associated with their use [217-219]. The problem with using a GFP-RFP FRET pair is the oligomerisation of red fluorescent proteins such as DsRed, which might affect the cellular localisation and behaviour of the tagged proteins. With the creation of mRFP1, a monomeric version of DsRed [220], the use of the GFP-RFP pair for FRET became more feasible. These two FPs have significant spectral overlap and can be used for FRET analysis (Figure 1.11) [211].

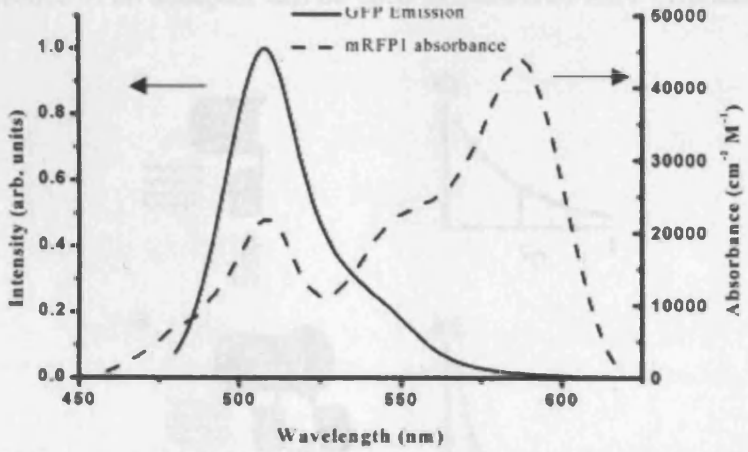


Figure 1.11: Overlap of the GFP emission and mRFP excitation spectra The respective emission and absorption spectra of EGFP and mRFP1 from protein extract solutions were measured and represented graphically. Adapted from [211].

1.8.4.2 Fluorescence lifetime imaging microscopy (FLIM)

Fluorescence lifetime imaging (FLIM) makes use of FRET between individual fluorophores. Other than FRET, where emission from the acceptor is measured, FLIM relies on the changes that occur in the lifetime of the donor fluorophore as a result of an acceptor being in its vicinity when excited. The fluorescence lifetime of a molecule is defined as the average time spent in the excited state after light absorption. The typical fluorescence lifetime ranges between picoseconds and nanoseconds [221]. Fluorescence lifetime is a parameter intrinsic to the fluorophore and is independent of relative fluorophore concentration or light path length. It is however, highly sensitive to processes taking place at the excited state such as FRET, which results in a decrease in donor lifetime (Figure 1.12) [221, 222]. By measuring the differences in donor lifetime in the absence or presence of an acceptor fluorophore, direct interaction between two fluorescently tagged proteins can be assessed.

Some of the advantages of using FLIM over FRET is that the concentration of acceptor does not need to be controlled (as long as it is in excess) and that it is insensitive to spectral bleed-through and fast photobleaching (a property of RFP that makes conventional FRET difficult). In addition, FLIM provides an internally calibrated FRET measurement (and thereby an internal control), and the differences in lifetime in the presence or absence of an acceptor can be used to measure FRET efficiency.

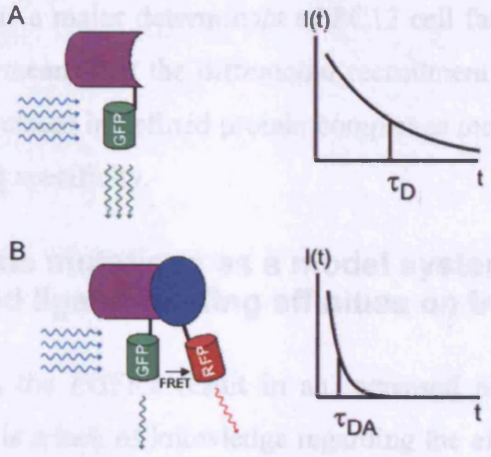


Figure 1.12: Diagrammatic representation of the decrease in donor fluorophore lifetime in the presence of FRET FLIM measures the average time a fluorophore remains in the excited stated upon absorption of a photon of excitation light. Upon excitation of the donor fluorophore (GFP) in the absence of a FRET acceptor (RFP) it emits light of a certain wavelength; its lifetime is represented by τ (A). When an acceptor fluorophore is brought into vicinity due to protein-protein interaction of the GFP- and RFP-tagged proteins, FRET takes place, which leads to emission from the acceptor and shortened lifetime of the donor fluorophore as indicated (B). Adapted from [223].

1.9 Objectives

One of the questions regarding signalling from receptor tyrosine kinases that still remains largely unanswered is how different receptors are able to maintain the integrity of specific signals that induce varied and defined cellular responses. Particularly the way in which specific recruitment of signalling proteins to a given receptor is achieved and the precise role that formation of unique multiprotein complexes plays in regulating activation of a defined set of downstream signalling pathways remain to be elucidated. The work presented herein aims to address these questions by using two different model systems.

1.9.1 The importance of precise protein recruitment to different tyrosine kinase receptors to generate specific cellular responses

Although numerous studies have focused on the differences in signals induced by various growth factors in PC12 cells, comparisons of the early signalling complexes induced by the EGFR, FGFR and TrkA are limited. Therefore PC12 cells were chosen as a model system to investigate the role of the assembly of different multiprotein complexes downstream of RTKs in regulation of specific cellular responses (namely differentiation or proliferation). It was chosen to focus on the recruitment of FRS2, Sos, Grb2 and Shc to the receptors, since all four proteins play a role in signalling downstream of TrkA, EGFR and FGFR. The recruitment of the Grb2/Sos complex to the receptor via Shc and/or FRS2 is an important regulator of the Ras/Raf/MEK/Erk1/2 pathway, which in turn is a major determinant of PC12 cell fate in response to growth factor stimulation. This means that the differential recruitment of these four signalling proteins and their involvement in defined protein complexes may be an important factor in determining signalling specificity.

1.9.2 Apert syndrome mutations as a model system to investigate the effects of altered ligand binding affinities on intracellular signalling

The Apert mutations in the FGFR2 result in an increased affinity for various FGF ligands. However, there is a lack of knowledge regarding the effects of these mutations on intracellular signalling events, particularly the early signalling events proximal to the activated receptor. These aspects make the Apert mutant receptors an ideal model system to investigate the effects of extracellular mutations on the recruitment of adaptor proteins into multiprotein complexes. The fact that the mutations primarily affect ligand binding, but are removed from the regions of the receptor that are involved in protein recruitment and activation of downstream signalling pathways, makes this system

particularly interesting. It allows investigation of the importance of ligand-receptor interaction in creation of specific downstream responses and additional effects of these mutations in the extracellular region on signalling downstream of the FGFR2. Mutations that alter the assembly of signalling complexes would be expected to change the ultimate signals generated. In the case of the Apert mutant receptors, the changes that occur on the outside of the cell are expected to alter the signalling events that are induced intracellularly upon ligand binding. The investigation will focus on whether these mutations simply cause increased signalling via the same pathways or whether they alter the way in which proteins are recruited to the receptor and the signals that are emanated as a result. This will further the understanding of the factors that are required in a receptor system to maintain highly regulated protein recruitment as well as integrity and specificity of the signals induced. Additionally, this investigation will allow analysis of the effects that disturbance of the receptor signalling system has on the specificity of downstream signalling events as well as cellular responses to FGF stimulation.

1.9.3 Investigation of the regulation of Shc recruitment to the FGFR2

In addition to addressing questions regarding the recruitment of multiple proteins into different protein complexes downstream of various receptors and the disturbance of protein recruitment and signalling specificity by mutations, it was chosen to specifically analyse the way that recruitment of a single protein to a receptor is mediated. To date, Shc has been implemented with a role in FGFR signalling, but its interaction with or recruitment to the FGFR has not been elucidated. It was therefore chosen to analyse the importance of the different Shc domains for its recruitment to the activated FGFR2. In addition to determining the mode of Shc recruitment to a specific receptor, this approach also allowed further insight into how intrinsic differences in terms of protein recruitment to different receptors might be mediated by providing direct binding sites for different proteins on a receptor.

1.9.4 Experimental approach

Changes in protein-protein interactions upon stimulation of a receptor are commonly assessed by immunoprecipitation and pulldown techniques. However, these approaches are limited to examination of the temporal changes, such as recruitment of proteins into a multiprotein complex, and cannot provide information about changes in protein localisation. Spatial changes in protein localisation intracellularly following receptor activation may be an important regulator of signalling specificity. It was therefore

chosen to use fluorescently tagged proteins to analyse the spatial as well as the temporal changes that occur in response to activation of various receptors. Co-localisation studies are useful to assess changes in localisation of two proteins to the same cellular compartment such as the plasma membrane. However, the resolution achieved by confocal microscopy is not high enough to assess changes in protein-protein interactions. Fluorescence lifetime imaging microscopy (FLIM) was therefore chosen to analyse changes in direct protein-protein interactions in addition to changes that occur in terms of their (co-)localisation. It was the technique of choice because the GFP-RFP pair was used and the rapid photobleaching of RFP makes measurements by traditional FRET methods difficult. Since FLIM does not rely on acceptor (RFP) emission, it allows accurate and detailed analysis of changes in protein interaction following recruitment to activated RTKs that may play an important role in regulating activation of specific and diverse signals. Analysis of changes in protein localisation and interaction intracellularly can be complemented with traditional western blotting and immunoprecipitation techniques to generate an overall picture of the importance of regulated protein recruitment to RTKs in the generation of signalling specificity.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemical reagents used throughout were purchased from Sigma, Melford or VWR unless otherwise stated.

2.1.2 Restriction and modification enzymes and polymerases

Calf intestinal phosphatase (CIP)	NEB
DpnI	Stratagene
Pfu Turbo DNA polymerase	Stratagene
Restriction endonucleases	NEB
RNAse	Invitrogen
T4 DNA ligase	NEB
Vent® DNA polymerase	NEB

2.1.3 Growth factors and ligands

Bovine pancreatic insulin	Sigma
Heparan sulphate salt	Sigma
Murine natural nerve growth factor 7S	Sigma
Recombinant human epidermal growth factor	Peprotech
Recombinant human fibroblast growth factor 2	Peprotech
Recombinant human fibroblast growth factor 9	R&D Systems

2.1.4 Kits and other materials

Enhanced chemiluminescence substrate (ECL)	Pierce Perbio
Glutathione resin	Novagen
Immobilon-P PVDF membrane 0.45μm	Millipore
Lipofectamine 2000 transfection reagent	Invitrogen
Protease inhibitor cocktail set III	Calbiochem
Protease inhibitor cocktail tablets (complete)	Roche
Protein A agarose	Sigma
Protein G agarose	Sigma
QIAprep spin miniprep kit	Qiagen
QIAquick gel extraction kit	Qiagen

Tissue culture flasks, dishes and multiwell plates	Helena Biosciences
Tissue culture flasks, dishes and multiwell plates	Sarstedt
Whatman filter paper 3MM	Fisher Scientific
XB-200 blue X-ray film	X-ograph

2.1.5 Bacterial and cell culture media and buffers

All growth media and buffers were prepared with distilled water from an Elga water purifier and autoclaved where appropriate.

2.1.5.1 Bacterial culture

Luria bertani (LB) broth

1.0% (w/v) tryptone

0.5% (w/v) yeast extract

0.5% (w/v) sodium chloride

The medium was autoclaved and appropriate antibiotics were added to a final concentration of 250μg/ml carbenicillin, 50μg/ml kanamycin, 50μg/ml tetracyclin (stock in 100% ethanol) or 50μg/ml chloramphenicol (stock in 100% ethanol).

NZY$^+$ broth

For transformation of the products of the mutagenised nicked circle plasmids (Stratagene Quikchange mutagenesis kit) a richer medium than LB broth is required.

1.0% (w/v) NZ amine (casein hydrolysate)

0.5% (w/v) yeast extract

0.5% (w/v) sodium chloride

pH 7.5

After autoclaving, the following filter sterilised supplements were added per litre of broth:

12.5ml of 1M magnesium chloride

12.5ml of 1M magnesium sulphate

20ml of 20% (w/v) glucose

2.1.5.2 Agar plates

Agar plates were created by the addition of 1.5% (w/v) agar to LB broth before autoclaving. Plates containing various antibiotics were prepared as described for the LB medium after the agar was cooled to 50°C.

2.1.5.3 Glycerol stocks

Glycerol stocks of all strains were created by addition of 300µl 50% glycerol in ddH_2O to 700µl of an overnight bacterial culture. The samples were frozen, stored at -80°C and used to streak cultures on agar plates when required.

2.1.6 Mammalian cell culture

All tissue culture media and supplements were purchased from Cambrex, Gibco or Invitrogen. Foetal calf serum (FCS) was supplied by Biosera and horse serum (HS) and plasma-derived (platelet poor) horse serum were obtained from Sigma.

HEK 293T complete growth medium

Dulbecco's modified Eagle's Medium (DMEM) with 4.5g/L glucose, 4mM L-glutamine, 10% FCS, 1% antibiotic/antimycotic solution (10,000units/ml penicillin, 10,000µg/ml streptomycin, 25µg/ml amphotericin B) and 50µg/ml gentamycin sulphate

PC12 complete growth medium

Dulbecco's modified Eagle's Medium (DMEM) with 4.5g/L glucose, 4mM L-glutamine, 10% HS, 5% FCS, 1% antibiotic/antimycotic solution and 50µg/ml gentamycin sulphate

Starvation medium

Complete growth medium without FCS for HEK 293T, or containing 0.1% HS in the case of PC12 cells

Dulbecco's PBS

1x PBS without calcium or magnesium, pH 7.3-7.7

Poly-D-lysine solution

0.01% poly-D-lysine hydrobromide (MW 70,000-150,000) in ddH_2O

Freezing medium
20% dimethylsulphoxide DMSO
80% FCS or HS

The freezing medium was diluted 1:1 with regular growth medium containing the cells to be frozen. Cells were frozen in cryotubes using a special cryo tub at -80°C and subsequently placed in liquid nitrogen for long-term storage.

2.1.7 Stock solutions and buffers

All buffers and solutions were prepared with distilled water from an Elga water purifier and autoclaved where appropriate.

Coomassie stain solution
0.2% (w/v) Coomassie Brilliant Blue R
40% (v/v) methanol
10% (v/v) acetic acid

Coomassie destain solution
10% (v/v) acetic acid
40% (v/v) methanol

6x DNA loading dye
60% glycerol
0.05% bromophenol blue

2x HBS
42mM Hepes pH 7.05
274mM NaCl
10mM KCl
1.5mM Na_2HPO_4
2% (w/v) glucose

2x/6x Laemmli loading buffer
100mM / 300mM Trizma hydrochloride pH 6.8
4% / 12% (w/v) SDS
20% / 60% (v/v) glycerol
0.6% bromophenol blue
10mM / 30mM DTT (added immediately before use)

Lysis buffer (bacterial)
1xTBS pH 8.0
0.1% Triton-X100

Before use 0.1mg lysozyme and one protease inhibitor cocktail tablet (Roche) were added per 10 ml lysis buffer.

Lysis buffer (mammalian)
20mM TrisHCl pH 7.5
138mM NaCl
1mM EGTA
20mM β-glycerophosphate
10% (w/v) glycerol
1mM sodium orthovanadate
20mM sodium fluoride

1% (v/v) protease inhibitor cocktail III (Calbiochem) was added directly before use.

Midiprep buffer P1
25mM Trizma hydrochloride pH 7.5
10mM EDTA
50mM glucose

Midiprep buffer P2
200mM NaOH
1% SDS

Midiprep buffer P3
3M potassium acetate pH 4.8

10x mounting solution (anti-fade agent)
1% (w/v) 1,4-phenylenediamine pH 9.5

1x mounting solution (anti-fade agent)
1ml 10x stock solution
9ml 50% glycerol in PBS
pH 7.5-8.0

Paraformaldehyde
10% (w/v) paraformaldehyde, pH 8.0 stock solution
4% (v/v) paraformaldehyde (10% stock diluted in PBS pH 8.0 for cell fixation)

1x PBS
10mM phosphate
2.7mM KCl
137mM NaCl
pH 7.4

Rapid screening buffer
5mM EDTA
10% w/v sucrose
0.25% w/v SDS
100mM NaOH
60mM KCL
0.05% Bromophenol blue

SDS-PAGE stacking gel buffer
0.5M Trizma hydrochloride pH 6.8

SDS-PAGE resolving gel buffer
3M Trizma hydrochloride pH 8.8

10x SDS-PAGE running buffer
1.9M glycine
250mM Trizma base
10% (w/v) sodium lauryl sulphate (SDS)

0.5x TBE
45mM Trizma borate
1mM EDTA
pH 8.3

10x TBS (Tris buffered saline)
500mM Trizma hydrochloride
1.5M sodium chloride
pH 7.4

1x TBS-T
100ml 10x stock solution
1mM EDTA
0.01% (v/v) Tween-20

10x Transfer buffer

1.9M glycine

250mM Trizma base

1x Transfer buffer (per litre)

200ml methanol

100ml 10x stock solution

900ml water

Western blot stripping buffer

62.5 mM Trizma hydrochloride pH 6.7

2% (w/v) SDS (200ml 10% (w/v) stock per litre after autoclaving)

0.7% (v/v) β-mercaptoethanol (added immediately before use)

2.1.8 Bacterial strains

Bacterial strain	Genotype	Use
XL1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})]	Plasmid vector propagation, cloning
BL21 (DE3)	E.coli B F^{-} *dcm ompT hsdS*($r_B^- m_B^-$) *gal*	Expression of recombinant protein
DH5α	F'phi80d*lacZ* delta(*lacZ*YA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (rk-, m k+) *pho*A *sup*E44 lambda-*thi*-1 *gyr*A96 *el*A1/F' *pro*AB+ *lac*IqZdeltaM15 Tn10(*tet*r)	Plasmid vector propagation, cloning , expression of recombinant protein

2.1.9 Cell lines

Name	Origin	Stably transfected with	Reference
HEK 293T	Human embryonic kidney fibroblasts transformed with adenovirus and SV40 T-antigen	-	ATCC: 293tsA1609neo
HEK 293T WT-FGFR2	Human embryonic kidney fibroblasts	WT-FGFR2-GFP monoclonal cell line	[224]
HEK 293T SW-FGFR2	Human embryonic kidney fibroblasts	S252W-FGFR2-GFP monoclonal cell line	[224]
HEK 293T PR-FGFR2	Human embryonic kidney fibroblasts	P253R-FGFR2-GFP monoclonal cell line	[224]
PC12	Rat adrenal phaeochromocytoma	-	ECACC: 88022401
PC12 WT-FGFR2	Rat adrenal phaeochromocytoma	WT-FGFR2-GFP monoclonal cell line	this work
PC12 SW-FGFR2	Rat adrenal phaeochromocytoma	S252W-FGFR2-GFP monoclonal cell line	this work
PC12 PR-FGFR2	Rat adrenal phaeochromocytoma	P253R-FGFR2-GFP monoclonal cell line	this work
PC12 Grb2-GFP	Rat adrenal phaeochromocytoma	Grb2-GFP monoclonal cell line	this work

2.1.10 Antibodies

Antibody (antigen/immunogen)	Host species	Source
phospho-Akt (synthetic phospho-peptide corresponding to residues surrounding Ser473 of mouse Akt)	Mouse	Cell Signaling Technology
Bek (epitope at the C-terminus of human FGFR2)	Mouse	Santa Cruz
Grb2 (amino acids 1-217 of rat Grb2)	Mouse	BD Transduction Laboratories
Grb2 (epitope at C-terminus of human Grb2)	Rabbit	Santa Cruz
Erk1/2 (synthetic peptide derived from rat p42 MAPK)	Rabbit	Cell Signaling Technology

phospho-Erk1/2 (synthetic phospho-peptide corresponding to region surrounding Thr202/Tyr204 of human p44 MAPK)	Mouse	Cell Signaling Technology
FRS2 (Peptide corresponding to residues 283-297 of human FRS2/SNT1)	Mouse	Upstate
GFP (recombinant *Aequorea victoria* GFP)	Goat	Rockland
GFP-HRP (amino acids 1-238 of *Aequorea victoria* GFP)	Mouse	Santa Cruz
PDGFRβ (extracellular domain of PDGRβ)	Mouse	Sigma
pY99 (phosphotyrosine)	Mouse	Santa Cruz
RFP (RFP fusion protein)	Rabbit	Chemicon International
Shc (residues 359-473 of human Shc)	Mouse	BD Transduction Laboratories
Shc (recombinant GST fusion corresponding to amino acids 366-473 of human Shc)	Rabbit	Upstate
Shc pY317 (peptide FDDPSpYVN-C corresponding to amino acids 312-319 of human p52 Shc)	Rabbit	Upstate
Shc pY239/pY240 (synthetic phosphopeptide correseponding to residues surrounding Y239/Y240)	Rabbit	Cell Signaling Technology
Sos1 (C-terminus of human Sos1, amino acids 1057-1178)	Rabbit	Santa Cruz
Anti-mouse secondary Ab, HRP conjugated	Goat	Sigma
Anti-rabbit secondary Ab, HRP conjugated	Goat	Sigma
Anti-goat secondary Ab, HRP conjugated	Guinea pig	Sigma

2.1.11 Plasmids and oligonucleotides

2.1.11.1 Original plasmids

Plasmid	Characteristics	Source
pcDNA 3.1 Hygro (+)	Mammalian expression vector, Amp^r, Hyg^r, CMV promoter, SV40 poly A	Invitrogen
pcDNA 3.1 (+)	Mammalian expression vector, Amp^r, Neo^r, CMV promoter, SV40 poly A	Invitrogen
pEGFP(N1/N2)	Mammalian expression vector allowing C-terminal GFP-fusion, Kan^r, Neo^r, CMV promoter, SV40 poly A	Clontech
pGEX4T-2	Bacterial expression vector allowing N-terminal GST fusion, Amp^r	Amersham Biosciences
pGEX2T	Bacterial expression vector allowing N-terminal GST fusion, Amp^r	Amersham Biosciences

2.1.11.2 Modified Plasmids

Plasmid	Insert	Reference/Source
RFPN	mRFP between NheI and HindIII sites of pcDNA 3.1 (+)	Ng, T., King's College London
RFPC	mRFP between NotI and XhoI sites of pcDNA 3.1 (+)	Ng, T., King's College London
WT-FGFR2-GFP	Full-length wild type FGFR2 in frame with GFP in pEGFP(N2)	[224]
S252W-FGFR2-GFP	Full-length S252W mutant FGFR2 in frame with GFP in pEGFP(N2)	[224]
P253R-FGFR2-GFP	Full-length P253R mutant FGFR2 in frame with GFP in pEGFP(N2)	[224]
pc3.1HygShc-RFPC	Human Shc amino acid 17-472 in HindIII site in frame with C-terminal mRFP	This work
pc3.1HygShc-RFPC-3F	Human Shc amino acids 17-472 containing Y239F, Y240F and Y317F mutations in HindIII site in frame with mRFP	This work

pc3.1HygShcΔPTB-RFPC	Human Shc amino acids 208-472 in HindIII site in frame with mRFP	This work
pc3.1HygShcR401A-RFPC	Human Shc amino acids 17-472 containing R401A mutation in frame with mRFP	This work
PTB(Shc)-RFPC	Human Shc amino acids 17-207 between EcoRI and EcoRV sites in frame with mRFP	This work
SH2(Shc)-RFPN	Human Shc amino acids 369-472 between BamHI and EcoRI sites in frame with mRFP	This work
pGEX2T-Shc	Human Shc amino acids 17-472 in frame with GST-tag	This work
pGEX2T-SH2(Shc)	Human Shc amino acids 369-472 in frame with GST-tag	This work
pGEX4T-2-PTB(Shc)	Human Shc amino acids 17-207 in frame with GST-tag	This work
Grb2-GFP(N2)	Human full-length Grb2 amino acids 1-217	This work
pGEX2T-Grb2	Human full-length Grb2 amino acids 1-217	This work

2.1.11.3 Oligonucleotides

All oligonucleotides read 5' to 3'.

Mutagenesis

CCA CCT GAC CAT CAG TTC TTT AAT GAC TTC CCG GGG

Forward primer for mutagenesis of Shc Y239 and Y240 to phenylalanine

CCC CGG GAA GTC ATT AAA GAA CTG ATG GTC AGG TGG

Reverse primer for mutagenesis of Shc Y239 and Y240 to phenylalanine

GCT TTT TGA TGA TCC CTC CTT TGT CAA CGT CCA GAA CC

Forward primer for mutagenesis of Shc Y317 to phenylalanine

GGT TCT GGC CGT TGA CAA AGG AGG GAT CAT CAA AAA GC

Reverse primer for mutagenesis of Shc Y317 to phenylalanine

GGA CTT CCT GGT AGC GGA GAG CAC G

Forward primer for mutagenesis of Shc R401 to alanine

GGT GCT CTC CGC TAC CAG GAA GTC C

Reverse primer for mutagenesis of Shc R401 to alanine

Gene amplification

GCG GGC TAT CCC TAT AAG CTT CCG GAC TAT GCA GG

Insertion of HindIII site N-terminal to Shc start codon

CTC TAG AGT CGC GGC GGT ACC GAT CAG TTT CCG

Insertion of KpnI site at Shc C-terminus

GCG CTT CAA ACA AAG CTT ATG GAA CCC

Insertion of HindIII site at position 206 of full-length Shc

CTC GGT ACC AAG CTT GAT ATC GAA TTC

Reverse primer for amplification of ShcΔPTB

CGC TGC TCG AAT TCA TAT GGG CCA GCT TGG G

Amplification of Shc PTB domain and insertion of EcoRI site

GGG GTC ACC AGT TTG ATA TCG TTC CTG AGG

Amplification of Shc PTB domain and insertion of EcoRV site

GCT CCG TCG ACG AAG CTT GCG GCC GC

Forward primer to introduce reading frame shift in Grb2 for creation of GFP-fusion

GCG GCC GCA AGC TTC GTC GAC GGA GC

Reverse primer to introduce reading frame shift in Grb2 for creation of GFP-fusion

Sequencing

TAG AAG GCA CAG TCG AGG

BGH reverse primer (pCR 3.1)

TAA TAC GAC TCA CTA TAG GG

T7 promotor forward primer

GCC CTC GCC CTC GCC

GFP/RFP reverse primer for sequencing junction of C-terminally tagged fusions

2.2 Molecular biological techniques

2.2.1 DNA preparation, analysis and manipulation

2.2.1.1 Preparation of DNA for manipulation

Small amounts of plasmid DNA for molecular biological manipulation were prepared using the QIAprep spin miniprep kit (Qiagen) according to the manufacturer's instructions. Approximately 10-20µg of plasmid DNA were recovered from a 5ml overnight culture in the XL1 Blue bacterial strain.

2.2.1.2 Preparation of DNA for transfection into mammalian cells

For high efficiency transfections of DNA into mammalian cells, large amounts of highly concentrated DNA were required. 100ml LB with the appropriate antibiotic were inoculated with 1ml of an overnight starter culture of XL1 Blue containing the target plasmid and grown overnight at 37°C in a shaking incubator. The bacterial cells were pelleted by centrifugation at 4000rpm for ten minutes and resuspended in 5ml buffer P1. 5ml of lysis buffer P2 were added. Precipitation of insoluble cell components was achieved by addition of 5ml neutralisation buffer P3.

The insoluble material was removed by centrifugation of the sample at 4000 rpm for 20 minutes, followed by transfer of the supernatant into a fresh tube. An equal volume of phenolchloroform isoamide alcohol was added and mixed by vortexing. The two layers were separated by centrifugation at 4000rpm for five minutes and the top layer was transferred to a fresh tube. Two volumes of cold 100% ethanol (30 ml) were added to precipitate the DNA which was subsequently pelleted by centrifugation at 4000 rpm for 30 minutes. The pellet was washed with 15ml cold 70% ethanol and the DNA was dried at room temperature.

To remove all RNA, the DNA was resuspended in 500µl TE with 10µg/ml RNAse and incubated at 37°C for one hour. Subsequently, 125µl 4M NaCl and 1ml of 13% PEG-800 were added. The DNA was left to precipitate on ice for two hours, subsequently pelleted by centrifugation at 13000rpm for 20 minutes and washed once with 1ml ice-cold 70% ethanol and once with 1ml 100% ethanol. The DNA was left to dry at room temperature and was resuspended in 200-400µl double distilled water.

2.2.1.3 Determination of DNA concentration

Double stranded DNA absorbs light at a wavelength of 260nm. To measure the concentration of PEG-precipitated DNA, the absorbance of a 1:100 dilution was

measured at 260nm using a Quartz cuvette. An OD_{260}=1 is equivalent to a DNA concentration of 50µg/ml.

For less concentrated samples such as Miniprep DNA or smaller volumes such as gel purified digested DNA, the DNA concentration was measured using 1µl of sample directly in a Nanodrop spectrophotometer.

2.2.1.4 Restriction digests

Restriction digests were set up according to the following standard dilutions and incubated in a waterbath at the appropriate temperature for two hours. The manufacturer's buffer and temperature recommendations for double digests were followed.

14µl DNA (plasmid DNA or PCR product)
2µl recommended buffer
2µl 10x bovine serum albumin
1µl (10,000-20,000U/ml) of each respective enzyme used

2.2.1.5 Dephosphorylation of DNA 5' ends

To prevent re-ligation of the vector when cut with only one restriction enzyme, 1µl (10,000U/ml) of calf intestinal phosphatase (CIP) was added to the restriction digest containing the backbone vector for the cloning reaction for one hour at 37°C. The removal of 5' phosphates prevents self ligation.

2.2.1.6 Separation of DNA fragments by gel electrophoresis

Double stranded DNA fragments from restriction digests were separated by gel electrophoresis on a horizontal 0.8% agarose gel in 0.5% TBE buffer containing ethidium bromide. 4µl of 6x DNA loading buffer were added to the restriction digests and the entire reaction was loaded in one well. A 1kb ladder (NEB) was used as a molecular size marker. Electrophoresis was carried out in 0.5x TBE at 80V for one hour. Separated bands were visualised on a UV transilluminator and excised from the gel.

2.2.1.7 DNA purification from agarose gel

The QIAquick gel extraction kit (Qiagen) was employed according to the manufacturer's instructions to purify the DNA fragments excised from agarose gels.

2.2.1.8 DNA ligation

Ligations to clone a restriction enzyme digested insert into the appropriately digested target vector were carried out according to a standard protocol as follows.

3µl backbone vector DNA
14µl insert DNA
2µl 10x T4 DNA ligase buffer (thawed on ice)
1µl (400,000U/ml) T4 DNA ligase

If the concentration of either insert of vector was significantly higher than that of the respective other component, the ratio of 3:14 was adjusted to 2:15 or 4:16 accordingly. The reaction mixture was incubated at room temperature for two hours and 10µl were used to transform 100µl competent XL1 Blue bacteria as described in section 2.2.3.2.

2.2.1.9 Rapid screening of ligation products

To easily assess whether any of a large number of colonies on the ligation plate contained a plasmid with an insert, a rapid screening method was employed in which bacteria are lysed and the entire DNA and RNA content is subjected to agarose gel electrophoresis.

Individual colonies were spotted on a fresh LB Agar plate containing the appropriate antibiotic and incubated at 37°C overnight. Cells from these streaks were picked, resuspended in 25µl rapid screening buffer and incubated for five minutes on ice followed by five minutes at 37°C. The membranous fraction was pelleted by centrifugation at 13000 rpm for five minutes and 15µl of the sample were run on a 0.8% agarose gel. The size of the plasmid was compared to the original plasmid without insert. Clones with lower electrophoretic mobility were chosen and screened for the correct insert by DNA preparation and restriction enzyme digest.

2.2.1.10 DNA sequencing

All constructs created by cloning or mutagenesis were sequenced with appropriate forward and reverse primers to ensure that reading frames of fusion proteins were intact and that site-directed mutagenesis gave positive results. 150fmol of pure plasmid DNA and 12-30fmol of each primer were delivered to the sequencing facility at the Wolfson Institute of Biomedical Research (University College London, UK) where sequencing was carried out using a Beckman Coulter CEQ 8000 genetic analysis system.

2.2.2 DNA amplification and mutagenesis

2.2.2.1 Polymerase chain reaction

To facilitate subcloning and to allow introduction of reading frame changes for various fusion tags individual cDNAs or fragments thereof were PCR amplified using primers that introduced new restriction sites. PCR reactions were set up as follows:

1 µl DNA template (10-25ng/µl)
5µl 10µM forward primer
5µl 10µM reverse primer
5µl 10x ThermoPol buffer (NEB)
5µl 10mM dNTP mix (2.5mM of each dATP, dCTP, dGTP and dTTP)
28µl ddH_2O
1µl Vent® DNA polymerase (NEB)

One reaction was also set up containing 2mM magnesium sulphate, as this may enhance product yield and in certain cases was necessary for any product to be obtained. Vent® DNA polymerase was chosen because it contains a proofreading enzyme and therefore introduces fewer mutations into the amplified DNA than for example Taq polymerase. The reactions cycles were:

Initial melting step:	95°C	5.0 minutes	
Melting step:	95°C	0.5 minutes	|
Annealing step:	55°C	0.5 minutes	| x 30 cycles
Extension step:	72°C	1.0 minutes	|
Final extension step:	72°C	5.0 minutes	

10µl of a PCR reaction were subjected to agarose gel electrophoresis and the DNA band corresponding to the PCR product was excised and purified as described. This DNA was subsequently subcloned by restriction enzyme digestion as described.

2.2.2.2 DNA mutagenesis

"Quikchange" mutagenesis kit

To mutate single residues and to remove or insert Stop codons the "Quikchange" mutagenesis kit from Stratagene was used. Pfu Turbo is a DNA polymerase with a very sensitive proofreading mechanism, which prevents the introduction of random mutations and allows amplification of entire plasmids up to 8kb. This allows

introduction of mutations at any chosen point within the gene of interest and prevents re-cloning of PCR amplified fragments being necessary. The manufacturer's protocol provided with the kit was altered slightly and the reaction mixtures set up contained:

1µl template (10-15ng/µl)
5µl 1µM forward primer
5µl 1µM reverse primer
5µl 10x Pfu Turbo buffer
2µl 10mM dNTPs (2.5mM of each dATP, dCTP, dGTP and dTTP)
31µl ddH_2O
1µl Pfu Turbo DNA polymerase (Stratagene)

The PCR cycles were set up as follows.

Initial melting step:	95°C	5.0 minutes	
Melting step:	95°C	0.5 minutes	|
Annealing step:	55°C	0.5 minutes	| x 20 cycles
Extension step:	68°C	20 minutes	|

Removal of parental plasmid DNA

After the PCR reaction, the sample contains both the intact parental plasmid without the mutations introduced and the newly created DNA with the mutations. The mutated DNA is nicked on both strands and is not methylated. This means that the parental DNA can be removed by DpnI digestion since this enzyme recognises its restriction site only when methylated, which means that it will leave the mutated DNA intact. 1µl (20,000U/ml) of DpnI enzyme was added to the PCR mixture and incubated at 37°C for two hours. 10µl of the reaction were transformed into competent XL1 Blue according to the manufacturer's instructions using NZY^+ broth instead of LB during the recovery phase.

2.2.3 Competent bacterial strains

2.2.3.1 Preparation of chemically competent bacterial strains

XL1 Blue

The XL1 Blue bacterial strain was used to propagate plasmid DNA. These cells were made competent using a rubidium chloride method. 100ml of LB containing tetracyclin were inoculated with 1ml of an overnight culture of the XL1 Blue strain. The culture

was grown until an OD_{595} of 0.42-0.45 was reached. Cells were collected by centrifugation at 4000 rpm for five minutes and resuspended in 30ml ice-cold buffer TFB1 (100mM RbCl, 50mM $MnCl_2$, 30mM potassium acetate, 50mM $CaCl_2$, 15% glycerol, pH 5.8). After a 90 minute incubation on ice, the cells were pelleted and resuspended in 4ml ice-cold buffer TFB2 (10mM MOPS, 10mM RbCl, 75mM $CaCl_2$, 15% glycerol, pH 6.8). Aliquots of 100µl were frozen at -80°C.

BL21(DE3)

To produce recombinant protein, the *E.coli* strain BL21(DE3) was used. The cells were made competent using calcium chloride. A 100ml culture of bacteria was grown as for the XL1 Blue strain, but after centrifugation, the pellet was resuspended in 40ml ice-cold 100mM $CaCl_2$ and incubated on ice for 20 minutes. This was repeated two more times. After the last incubation, cells were resuspended in 4ml 100mM $CaCl_2$ with 10% glycerol, aliquoted into 100µl aliquots and stored at -80°C.

2.2.3.2 Transformation of competent cells

DNA was introduced into bacterial strains by chemical transformation. Competent bacteria were thawed on ice and plasmid DNA (0.5µl), ligation or mutagenesis products (10µl) were added. The tube was tapped lightly to mix in the DNA and incubated on ice for 30 minutes. Bacteria were heat-shocked at 42°C for 45 seconds and placed on ice for a further two minutes. 900µl of warm LB were added and samples were recovered at 37°C for 60 minutes. In the case of ligation of mutagenesis products, cells were pelleted by centrifugation at 13000rpm for 30 seconds, resuspended in 100-200µl LB and spread on a pre-warmed LB plate containing the appropriate antibiotic for selection. When pure plasmid DNA was transformed, 100µl were spread on the plate directly after recovery.

2.3 Cell biological techniques

2.3.1 General techniques

All tissue culture work was carried out aseptically in a filter flow cabinet (HeraSafe, Heraeus). All cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C using special tissue culture plasticware. Cell stocks were frozen in medium containing 50% FCS and 10% DMSO and stored in liquid nitrogen. When bringing up stocks from the frozen state, aliquots were thawed quickly in a 37°C waterbath and cells were transferred to 20ml fresh, pre-warmed respective growth medium and allowed to

recover for 2-4 days. Stably transfected HEK 293T and PC12 cells were not used beyond passage 15 and 8 respectively to avoid alterations in expression levels.

2.3.2 Coating of dishes and slides

To allow adherence of loosely attaching cells such as the PC12 cell line to the plastic surface of the tissue culture dishes and glass coverslips, these had to be coated with poly-D-lysine. Poly-D-lysine was chosen as a substratum to avoid the breakdown of poly-L-lysine that may occur by some cell types and result in their overload with L-lysine. Dishes were covered with the following volumes of 0.01% poly-D-lysine hydrobromide (w/v), incubated for four hours at room temperature and sterilised using a UV lamp. Following this, the poly-D-lysine solution was removed by aspiration and the dishes were dried at 37°C for several hours. Dishes were always prepared the day before use.

Diameter of dish	Surface area (mm^2)	Volume of poly-D-lysine
10cm	7900	5.0ml
6cm	2800	3.0ml
3.5cm	1000	1.5ml

Poly-D-lysine slides were prepared by incubating glass coverslips in 0.01% poly-D-lysine hydrobromide solution (w/v) at 37°C overnight and dried at 50°C for several hours. The slides were stored at room temperature for several weeks.

2.3.3 HEK 293T cells

HEK 293T cells are epithelial cells of human embryonic kidney origin. They are a derivative of the HEK 293 cell line that has been additionally transformed with the SV40 large T-antigen. More recently it has been reported that they may actually be of neuronal origin, as they express a number of neuronal cell markers [225]. They have been transformed with adenovirus and additionally contain the SV40 large T-antigen. HEK 293T cells were grown in DMEM growth medium and cells were passaged approximately 1:25 every 2-3 days by washing the monolayer once with Dulbecco's PBS, then once with Versene/EDTA 1:5000 (Invitrogen), incubating cells at 37°C for 5 minutes and resuspending them in complete growth medium.

2.3.4 PC12 cells

The PC12 rat pheocytochroma cell line was grown in DMEM growth medium and cells were subcultured at a ratio of approximately 1:10 – 1:15 every 3-4 days by transferring

part of the cells in suspension into a new tissue culture flask containing fresh medium. Clumps were broken up by pipetting along the sides and bottom of the tissue culture flasks.

2.3.5 Transfection

2.3.5.1 Lipofection

HEK 293T cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Briefly, for a 3.5 cm dish of 80% confluent cells, 10µl of the transfection reagent were diluted in 250µl Optimem serum free medium and incubated at room temperature for five minutes. 5µg of plasmid DNA were also diluted in 250µl Optimem and mixed with the Lipofectamine 2000 reagent after the incubation time by gentle pipetting. The mixture was incubated at room temperature for a further 20 minutes and added dropwise to the cells in complete medium, mixed by gentle rocking of the dish and incubated overnight.

For stable transfections, transfectants were selected in 400µg/ml hygromycin (Invivogen) or 800µg/ml geneticin (G418) (Invivogen) for two weeks with regular medium change every 3-4 days. Individual clones expressing the desired target protein were isolated by dilution cloning in 96-well plates. Stable cell lines (pools and clones) were maintained in 200µg/ml geneticin or 100µg/ml hygromycin.

2.3.5.2 Electroporation

PC12 cells were transfected by electroporation using a Biorad Gene Pulser II. $10x10^6$ cells were collected by centrifugation at 1100 rpm for 5 minutes, resuspended in 800µl Dulbecco's PBS and mixed with 40µg purified plasmid DNA by gentle pipetting. After a five minute incubation period on ice, cells were transferred to a 4mm electroporation cuvette and pulsed at 300V and 960µF. Cells were allowed to recover for ten minutes on ice, followed by a wash in 10ml room temperature PBS and incubation for at least 24 hours in pre-warmed complete growth media.

For the selection of stable cell lines, antibiotics (800µg/ml geneticin or 600µg/ml hygromycin) were added after the recovery period and the medium was changed every 3-4 days. Clonal cell lines were established by dilution cloning in 96-well plates. Stably transfected cell lines were maintained in medium containing 100µg/ml hygromycin or 200µg/ml geneticin.

2.3.5.3 Calcium phosphate precipitation

HEK 293T cells were transfected on a large scale using the calcium phosphate precipitation method. For a 10cm dish containing 10ml growth medium, 15µg DNA were diluted in a total volume of 438µl ddH_2O. 62µl 2M calcium chloride solution were added. While constantly vortexing at a low setting, 500µl of 2x HBS were added dropwise to the DNA solution. Within two minutes of 2x HBS addition, the mixture was added dropwise to the cells, taking care to spread the reagent well. Cells were incubated overnight and subsequently used for experiments.

2.3.5.4 Selection and clonal selection

After selection of stable clones for two weeks and assessment of target protein expression by fluorescence and/or western blotting techniques, dilution cloning was carried out to isolate individual clones of cells expressing the desired target protein. The cells were diluted in growth medium containing the appropriate antibiotics. Cells were then plated in 96-well plates and allowed to grow for several days until colonies could be identified. Plates were screened for wells containing individual colonies. These were picked, expanded and their expression compared by fluorescence microscopy and/or western blotting.

2.3.6 PC12 differentiation assay

For the differentiation assay PC12 cells were resuspended in Versene/EDTA and incubated at 37°C for 5 minutes to break up clumps and obtain single cells. $1x10^5$ cells were seeded in a 3.5cm dish previously coated with poly-D-lysine in growth medium. After 16-24 hours the medium was changed to differentiation medium containing 0.1% HS and 20ng/ml FGF9. Cells were assayed for neurite outgrowth after 4, 8, 24, 48 and 72 hours. 400-500 cells were counted and cells with neurites greater than twice the cell diameter were scored as positives. The differentiation medium was changed every 24 hours. Images of random fields of view at the indicated times were obtained using a digital camera fitted to a Zeiss Axiovert 25 microscope and operated using Openlab 4.0.1 software.

2.4 Protein manipulation and analysis

2.4.1 Stimulation and lysis of mammalian cells

For stimulation assays, cells were plated in 3.5, 6 or 10cm dishes at appropriate densities and allowed to reach approximately 80% or 60-70% confluency for HEK 293T

or PC12 cells respectively. Cells were serum-starved for 18 hours by replacing the growth medium with serum-free DMEM (HEK 293T) or DMEM containing 0.1% HS (PC12). Serum-starved cells were stimulated with various growth factors (reconstituted from lyophilised powder according to the manufacturer's instructions and diluted appropriately in Dulbecco's PBS). After addition of growth factor, dishes were placed in the incubator for various stimulation periods. Subsequently all medium was removed and cells were washed once in ice-cold PBS (1-3ml depending on dish diameter). Dishes were placed on ice and the cells were lysed in ice-cold lysis buffer by scraping with a rubber policeman.

Dish diameter	3.5cm	6cm	10cm
Volume lysis buffer	150µl	300µl	1000µl

Lysates were collected and the insoluble fraction was removed by centrifugation at 13000rpm for 20 minutes. The supernatant was collected in fresh Eppendorf tubes and either directly subjected to SDS-PAGE as whole cell lysate or used in immunoprecipitation or pull-down experiments.

2.4.2 Determination of protein concentration using the Bradford assay

To ensure that the same amount of protein was subjected to immunoprecipitation, pull-down or SDS-PAGE electrophoresis, the total protein concentration of lysates was determined with the Bradford protein assay. This assay works on the principle of an absorbance shift when the Coomassie Brilliant Blue G-250 dye binds to arginine and to a lesser extent histidine, lysine, tyrosine, tryptophan, and phenylalanine contained in the proteins of a sample. The bound, anionic form portays an absorbance shift to 595nm compared to 470nm for the unbound, cationic form. The protein concentration was calculated according to the formula $y=0.0496x+0.005$, where y is OD_{595} and x is the concentration in mg/ml. This was determined by creation of a standard curve using bovine serum albumin at various concentrations.

2.4.3 Gel electrophoresis

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [226]. The addition of sodium-dodecyl-sulphate (SDS) to the protein sample denatures the protein and causes all proteins to acquire an overall negative charge that is related to the number, but not the composition of amino acids in

the protein. This means that proteins can be separated on the basis of their molecular weight alone.

The protein gels used had dimensions of 6cm x 10cm and a thickness of 1.5mm. They were made up as follows to achieve separation of proteins of different sizes. Depending on the size of the proteins to be analysed, 8% or 10% acrylamide gels were used. The resolving gel was overlaid with a 4% stacking gel into which the well combs were inserted.

Component	**8% resolving gel Volume (ml)**	**10% resolving gel Volume (ml)**	**4% stacking gel Volume (ml)**
3M Tris-HCl pH 8.8	1.875	1.875	-
0.5M Tris-HCl pH 6.8	-	-	1.500
30% acrylamide/ 0.8% bisacrylamide	3.975	4.970	0.750
10% SDS	0.150	0.150	0.060
ddH_2O	8.775	7.780	3.000
10% ammonium persulphate	0.113	0.113	0.150
TEMED	0.015	0.015	0.003

Equal amounts of whole cell lysate for each sample were mixed with 6x Laemmli loading dye and boiled for five minutes in a heating block. Samples were subjected to electrophoresis at 200V and a maximum of 60mAmp per gel for one hour. Gels were either Coomassie stained or subjected to transfer onto PVDF membrane as described.

To determine the molecular weight of proteins on the gel or the subsequent western blot membrane a pre-stained protein marker (Biorad) was run alongside the samples. The marker contained pre-stained proteins of 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10kDa.

2.4.4 Coomassie staining of gels

Coomassie staining is a relatively low sensitivity method (detection range of 100-1000ng of protein) to visualise proteins on an SDS-PAGE gel, that is based on the Comassie Brilliant Blue R dye that binds non-specifically to all proteins. For protein staining, SDS-PAGE gels were incubated in stain solution for 4-5 hours or overnight with gentle agitation. To remove non-specifically bound dye from the gel, it was placed

in destain solution with gentle agitation until individual bands could be clearly visualised.

2.4.5 Western blotting

2.4.5.1 Transfer of proteins onto PVDF membrane

Western blotting refers to a procedure by which proteins are transferred from an SDS-PAGE gel onto a PVDF membrane. The positioning of individual protein bands is retained and the membrane can be probed with various primary antibodies to detect the presence, positioning and modification state of proteins.

Before use, the PVDF membrane was moistened in methanol, to allow efficient attachment of proteins to the membrane during transfer, and subsequently washed in transfer buffer. A blotting 'sandwich' containing the gel and membrane was assembled using a Biorad blotting module for the Mini Protean gel apparatus according to standard procedures. Transfer of proteins was carried out at 250mAmp for two hours in transfer buffer.

2.4.5.2 Protein staining after transfer using Ponceau stain

To detect whether even transfer of proteins onto the membrane has occurred, the membrane was stained using 0.1% (w/v) Ponceau S in 5% acetic acid. The excess stain was removed from the membrane by quick successive washes in ddH_2O. Once the protein bands were visualised, the dye was removed by quick successive washes in ddH_2O and a ten minute wash in TBS-T.

2.4.5.3 Immunoblotting

After protein transfer, the membrane was treated with blocking buffer (5% non-fat dried milk in TBS-T or 3% bovine serum albumin (BSA) in TBS-T depending on the antibody manufacturer's recommendation) for one hour. This ensures that all empty space between the proteins transferred from the gel are occupied by non-specific protein, to prevent antibodies attaching to the membrane non-specifically.

Membranes were generally incubated with 1µg of primary antibody overnight at 4°C in 5ml blocking buffer, with the exception of the pY99 anti-phosphotyrosine antibody, which was added for two hours at room temperature. Membranes were subsequently washed three to four times for ten minutes in 50-100ml TBS-T on a horizontal shaking platform. Following the washes, the appropriate horseradish-peroxidase-tagged antibody (anti-goat, anti-rabbit or anti-mouse) was added in a 1:1000 dilution in 5ml blocking buffer and incubated for two hours at room temperature. Membranes were

washed three to four times for ten minutes and bands were detected using enhanced chemiluminescence.

2.4.5.4 Detection of protein bands by enhanced chemiluminescence (ECL)

The secondary antibodies used to detect the bands on the western blot were tagged with horseradish-peroxidase. This enzyme is able to carry out a reaction that creates chemiluminescence when the two substrates of the enhanced chemiluminescence (ECL) kit (Pierce) are mixed and added to the membrane. The membrane was incubated with the ECL substrate for one minute according to the manufacturer's instructions. Detection was carried out using X-ray film in a dark room (exposure time between ten seconds and ten minutes depending on intensity of bands for various antibodies) or a Fujifilm Las-1000 phosphoimager operated by Image Reader Las 1000 software.

2.4.6 Immunoprecipitation

Whole cell lysates were subjected to immunoprecipitation with selected primary antibodies to investigate the presence and changes in protein complexes upon stimulation of cells with growth factors. Depending on the type of cells used, the whole cell lysates from a 10cm tissue culture dish contained various amounts of protein. Generally, 1.5-3.5mg of total protein in 1ml were used for immunprecipitation. 1-2μg of the chosen primary antibody was added, followed by incubation overnight at 4°C on a rotating wheel to allow formation of immuno-complexes. After 16 hours, 50μl 50% Protein A or Protein G agarose slurry in PBS were added for an additional four hours. Subsequently, the beads were washed four times with 1ml lysis buffer by removing the supernatant after centrifugation at 13000rpm for 30 seconds and replacing it with fresh lysis buffer. After the last wash, the beads were dried completely by careful removal of all supernatant using a thin pipette tip and were resuspended in 45μl 2x Laemmli loading buffer. The samples were boiled and subjected to SDS-PAGE as described.

2.4.7 GST-tagged protein expression

GST-tagged Grb2 and $p13^{suc-1}$ were expressed in the DH5α bacterial strain. 500ml of LB were inoculated with 5ml of overnight starter culture in the presence of the appropriate antibiotics and grown until they reached mid-log phase (OD_{595}=0.5). Cells were induced by addition of 0.5mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for four hours and subsequently pelleted by centrifugation at 5000 rpm for 15 minutes. Pellets were lysed in bacterial lysis buffer by sonication (three bursts of 20 seconds). The insoluble material was removed by centrifugation at 13000 rpm for 20 minutes. The

cell lysate was incubated with glutathione beads (provided in 20% ethanol and washed extensively in PBS and once in bacterial lysis buffer) overnight on a rotating wheel. The beads were washed four times with bacterial lysis buffer and stored at -20°C after the addition of an equal volume of glycerol.

GST-tagged full-length Shc and the Shc SH2 and PTB domains respectively were expressed in the BL21(DE3) bacterial strain and provided by Dr. R. George. The SH2 and the PTB domains were stable for storage at -20°C in glycerol, but full-length Shc was made directly before use to avoid protein degradation.

2.4.8 GST-tagged protein pulldown experiments

The volumes of beads with attached Grb2-GST or $p13^{suc-1}$ required for a pulldown assay was assessed by SDS-PAGE. If only a small volume was required, the beads were mixed with additional glutathione beads to enable visualisation. 1.5-3.5mg of total protein in 1ml were added to the beads and incubated overnight at 4°C on a rotating wheel to allow complex formation. The beads were washed four times with 1ml lysis buffer by removing the supernatant after centrifugation at 13000rpm for 30 seconds and replacing it with fresh lysis buffer. After the last wash, the beads were dried completely and were resuspended in 45µl 2x Laemmli loading buffer. The samples were boiled and subjected to SDS-PAGE as described.

2.5 Confocal microscopy analysis

2.5.1 Cell seeding and stimulation

HEK 293T cells were seeded on glass coverslips and allowed to attach/spread for 24 hours. PC12 cells were resuspended after centrifugation for 5 minutes at 1000rpm to break up clumps and obtain as many single cells as possible. They were seeded on poly-D-lysine coated slides and allowed to attach for 24 hours. Cells were serum-starved and stimulated as described previously.

2.5.2 Cell fixation and slide preparation

2.5.2.1 Fixation

After stimulation, the coverslips were washed once in ice-cold PBS and cells were fixed by the addition of 4% (w/v) paraformaldehyde for 20 minutes. The coverslips were washed five times in 1.5ml PBS pH 8.0 to remove all traces of paraformaldehyde.

2.5.2.2 Preparation of slides

After extensive washing, the coverslips were submerged in 0.5x mounting solution in PBS for a few seconds and subsequently placed upside down on a glass slide on a drop of 1x mounting solution. The excess liquid was removed by blotting with tissue paper and the slides were sealed using clear nail varnish. Slides were stored at 4°C for up to two days or at -20°C for longer storage, although this usually impaired fluorescence and was avoided where possible.

2.5.3 Confocal microscopy

Slides were analysed using a Leica TCS SP2 system with a 63x oil immersion objective. GFP was excited at 488nm using an argon visible light laser, RFP was excited at 568nm using a krypton laser. Their emission was detected using 514/10nm and 595/10nm band selection respectively. Mid-sections of the cells imaged were chosen to avoid interference from the attachment of the cell to glass. Fluorescence images (image size 512x512 pixels) were collected using a photomultiplier tube interfaced to an Intel Pentium II system running the Leica TCS NT control software. The images obtained were analysed using the Leica LCS lite software and co-localisation fluorescence intensity graphs were generated using the quantification function of the software. Approximately 10 cells were analysed for each image presented; the images shown are representative of the overall trend observed.

2.5.4 Fluorescence lifetime imaging microscopy (FLIM)

FLIM analysis was carried out using a Leica TCS SP2 inverted microscope set-up with a 63x water immersion objective, which was adapted for Time-Correlated Single-Photon Counting (TCSPC) FLIM with a Becker & Hickl SPC 830 card using 64 or 256 time channels in a 3 GHz, Pentium IV, 1GB RAM computer. The samples were excited using a femtosecond Titanium Sapphire laser (Coherent Mira, repetition rate 76MHz) that was pumped by a 6.5W solid state laser (Coherent Verdi V6). Images (512x512 pixels, reduced to 256x256 on the FLIM system) were obtained with a line scan speed of 200Hz. Two-photon excitation was carried out with a wavelength of 900nm and fluorescence was detected through a 525+/-25nm interference filter using a cooled PMC100-01 detector (Becker & Hickl, based on a Hamamatsu H5772P-01 photomultiplier). The fluorescence decays obtained were fitted using a single exponential decay model with Becker & Hickl SPCImage software v2.8.3. GFP

fluorescence lifetimes were portrayed in false colour maps. The images presented are representative of at least five independent fields of view analysed.

Chapter 3

Differences in protein recruitment to the EGFR, FGFR and TrkA regulate signal specificity

3.1 Introduction

Signalling pathways are often represented and viewed in a linear fashion. However, several factors have been identified that raise the question of how specificity in tyrosine kinase receptor-mediated signalling is achieved: (i) individual protein-protein interactions do not confer sufficient specificity to allow signalling to take place in the form of linear pathways, (ii) many RTKs recruit and activate the same signalling proteins and (iii) the same downstream targets such as Erk1/2, PLCγ or PI3K and various transcription factors are activated. These points indicate that linearity causes a number of problems in terms of achieving biological specificity and highlight the need to elucidate how signal crossover can be avoided and how specific responses can be achieved. The formation of multiprotein complexes downstream of activated RTKs has been implicated as an important mechanism for regulating specificity in RTK signalling [126, 135]. The work presented in this chapter will focus on the regulation of downstream responses to stimulation with different growth factors by recruitment of adaptor proteins into unique signalling complexes.

PC12 cells are a model system commonly used to compare signalling pathways initiated by different RTKs (for example [16, 67, 227, 228]). Previous work has indicated that the assembly of different multiprotein complexes is important for regulation of whether cells undergo proliferation or differentiation. Proliferation is primarily characterised by the transient activation of the Erk1/2 pathway in response to EGF or insulin stimulation. In contrast, differentiation requires prolonged activation of this kinase initiated by stimuli such as NGF or FGF [16]. Thus although these different tyrosine kinase receptors share many components on the qualitative level, quantitative differences between the signalling pathways are important in the regulation of cellular responses. More recently it has been shown that although both physiological processes rely on activation of Erk1/2, differential activation of the Raf/MEK/Erk1/2 pathway via Ras or Rap1 due to recruitment of different complexes to the plasma membrane upon EGFR or TrkA activation respectively is an important regulator of specific downstream responses [67, 228].

As a result of the clear physiological differences in response to stimulation with different growth factors, PC12 cells are an excellent model system to investigate the way in which cells regulate specific outcomes in response to activation of various

tyrosine kinase receptors. Although several independent studies have shown the importance in transient versus sustained Erk1/2 activation in the decision to undergo proliferation or differentiation, there is a lack of studies comparing the formation of early signalling events induced by the activation of the FGFR, EGFR and TrkA. Many studies have focused on events distal to the activated receptor, namely Erk1/2 activation and the role of the two different small GTPases Ras and Rap1 in this process in response to growth factor stimulation [67, 228-230]. However, the proximal signalling events initiated by the various receptors have not been fully elucidated. Altogether, the PC12 cell line provides an interesting model system to investigate the assembly of different early signalling complexes downstream of the three different receptors (EGFR, FGFR and TrkA) and their importance in regulating specific cellular outcomes.

To investigate the differences in early signalling complex formation downstream of the EGFR, FGFR and TrkA, this work focused on the signalling proteins Shc, Sos, Grb2 and FRS2. All four proteins have been reported to play a role downstream of each of the three receptors. However, the contribution of each component to the activation of Erk1/2 signalling varies depending on which receptor is activated. This provides an interesting basis to investigate the role that differences in protein recruitment play in activating specific downstream responses.

Techniques that involve isolation of protein complexes from cells mainly focus on the temporal regulation of signalling by complex assembly, since no information on the localisation of proteins and their binding partners can be obtained. However, the spatial distribution of signalling complexes provides another important point of regulation, because cellular localisation may affect the signal that emanates from various protein complexes. Indeed, cellular compartmentalisation of signalling molecules is an important factor in generating specific signals and avoiding signal crosstalk (reviewed in [145]). Changes in cellular localisation of adaptor proteins such as Shc, FRS2, Grb2 and Sos in response to PC12 stimulation with EGF, FGF or NGF have not been investigated previously. However, the identification of differences in spatial distribution of these proteins and interactions with each other in response to activation of different receptors would provide further insight into the way in which specific downstream responses are initiated, controlled and downregulated. The work presented in this chapter highlights temporal differences in protein recruitment to unique signalling complexes downstream of the EGFR, FGFR and TrkA. Further it presents the basis for

investigation of spatial differences in terms of protein localisation and interactions using fluorescently tagged signalling proteins.

3.2 Results

3.2.1 Different patterns of Erk1/2 activation by EGF, NGF and FGF2

It is a widely accepted dogma that NGF and FGF lead to sustained Erk1/2 activation, whereas EGF leads to a more transient stimulation of this downstream kinase. However, most published studies only compared two of these growth factors. Additionally, variations in the concentrations of growth factors used and different ways of measuring Erk1/2 activation make comparison between studies difficult. Consequently the effect of stimulation with equal amounts of all three growth factors on Erk1/2 phosphorylation was assessed over the time course of two hours (Figure 3.1). A concentration of 20ng/ml of each growth factor was found to induce comparable levels of Erk1/2 phosphorylation after stimulation of all three receptors for ten minutes and was chosen for subsequent experiments (data not shown). The results obtained from time course stimulation of PC12 cells with all three growth factors were in agreement with previous reports. Both NGF and FGF2 led to sustained phosphorylation of Erk1/2, which is representative of its prolonged activation (Figure 3.1). Even though the phosphorylation declined slightly around 30 minutes after stimulation, the return to near-basal levels of Erk1/2 phosphorylation that was observed after prolonged EGF stimulation did not occur (Figure 3.1B). EGF stimulation on the contrary led to more transient Erk1/2 activation. After 30 minutes of stimulation, the levels of phosphorylated Erk1/2 returned to a low level and by 60-120 minutes had nearly returned to basal levels.

More subtle differences in Erk1/2 phosphorylation downstream of the activated TrkA, EGF and FGF receptors were also observed. The onset of Erk1/2 activation following EGF stimulation was much faster (i.e. a large amount of phospho-Erk1/2 was already present after 2 minutes of stimulation) than after NGF or FGF2 stimulation (Figure 3.1B: inset graph). Thus overall, EGF-stimulated Erk1/2 activation was quicker and more transient. NGF- and FGF2-stimulated Erk1/2 activation portrayed a slightly slower onset but was sustained over several hours.

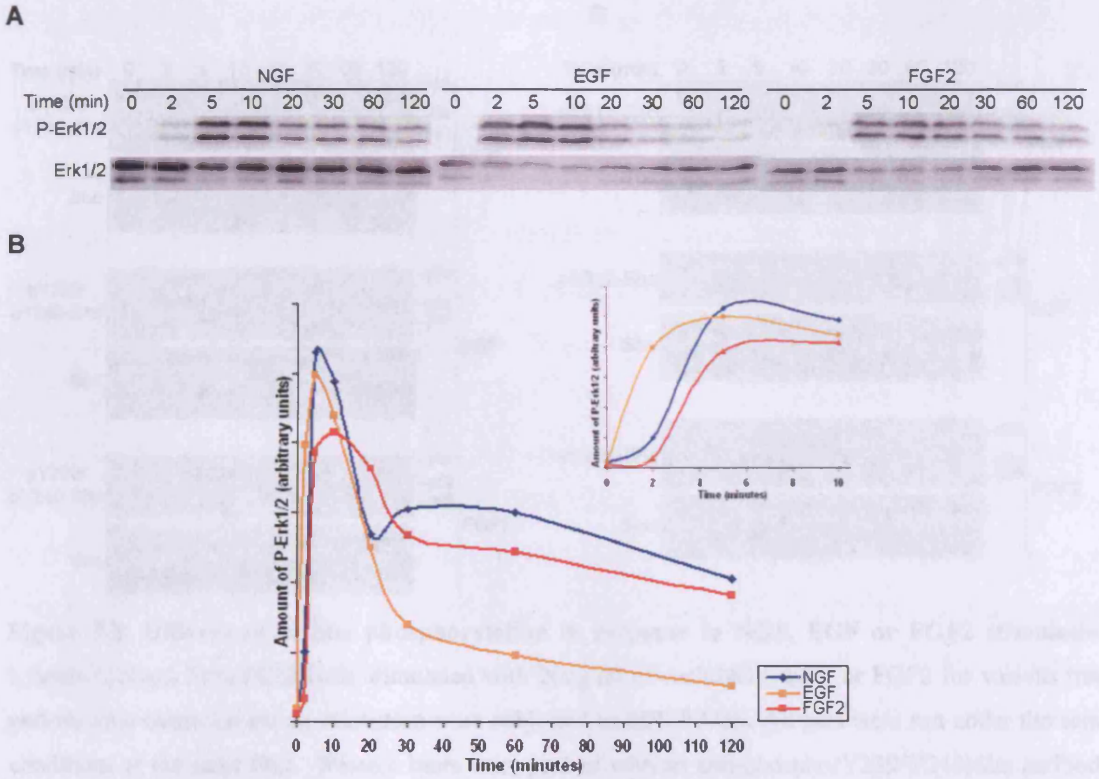


Figure 3.1: Erk1/2 phosphorylation in response to PC12 stimulation with NGF, EGF or FGF2 PC12 cells were serum-starved overnight and stimulated with 20ng/ml of each growth factor for various time periods. Cells were lysed and 200µg of whole cell lysate were subjected to SDS-PAGE. The Western blot was probed with an anti-phospho-Erk1/2 antibody, stripped and re-probed with an anti-Erk1/2 antibody as loading control (A). The intensity of the phospho-Erk1/2 bands were quantified using densitometry and the data represented graphically. The inset graph shows the early time points of the same graph in an expanded fashion (B). The results are representative of three independent experiments.

3.2.2 Shc is differentially phosphorylated following NGF, EGF and FGF2 stimulation

Full-length Shc contains two Grb2-binding sites around phosphorylated tyrosine residues 239/240 and 317 in the collagen-homology 1 (CH1) domain. Both positions form docking sites for the Grb2 SH2 domain when tyrosine phosphorylated but are also involved in activation of divergent, non-redundant signalling pathways [72, 198, 199]. Since Sos recruitment via Grb2 is an important step in Ras (and hence Erk1/2) activation, the effects of stimulation with different growth factors on Shc phosphorylation was investigated using specific antibodies raised against the two different tyrosine phosphorylated motifs.

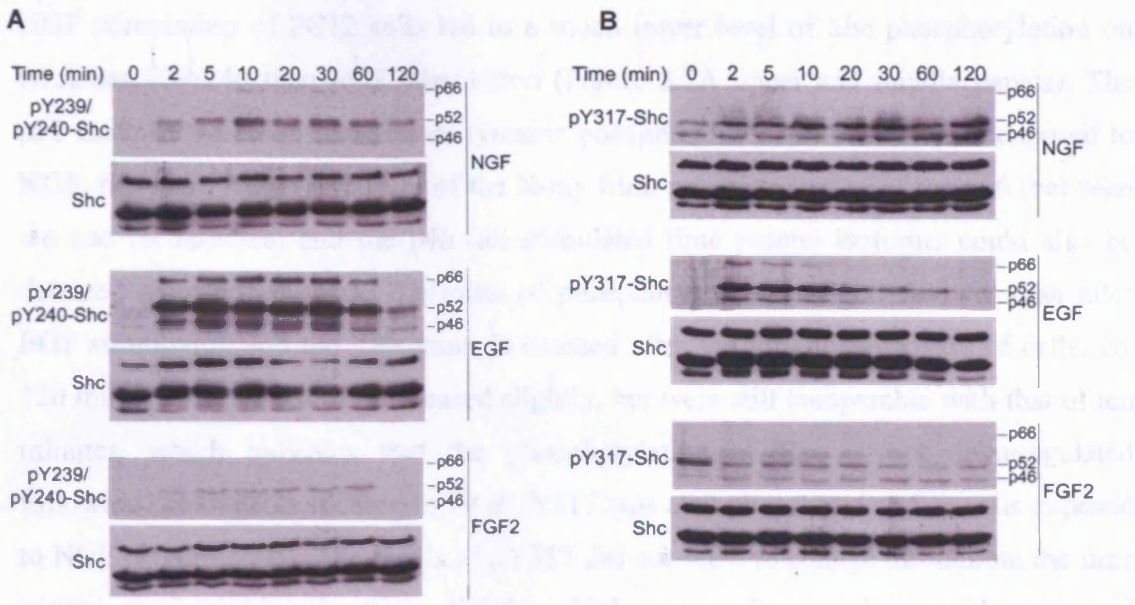


Figure 3.2: Differences in Shc phosphorylation in response to NGF, EGF or FGF2 stimulation
Lysates (200µg) from PC12 cells stimulated with 20ng/ml of each NGF, EGF or FGF2 for various time periods after overnight serum-starvation were subjected to SDS-PAGE. All gels were run under the same conditions at the same time. Western blots were probed with an anti-phospho(Y239/Y240)Shc antibody (A) or and anti phospho(Y317)Shc antibody (B). The blots were stripped and re-probed with an anti-Shc antibody to control for equal loading. The western blots in (A) were exposed for the same amount of time. The western blots in (B) were exposed for different periods of time to highlight the temporal changes for each growth factor rather than the direct comparison between the growth factors. The results are representative of two independent experiments in each case.

Both Grb2 binding sites on all three Shc isoforms are highly phosphorylated after stimulation of PC12 cells with EGF. Interestingly, phosphorylation of the p46 and the p66 isoforms was downregulated faster than that of the p52 isoform (Figure 3.2). Phosphorylation of both the Y317 and the Y239/Y240 sites was downregulated after 120 minutes of stimulation, which is in accordance with the more transient nature of the Erk1/2 signal in EGF-stimulated cells. Y317 was dephosphorylated more quickly than Y239/Y240. Y317 has been reported to be the main Grb2 binding site, and its downregulation by 30 minutes of EGF stimulation is in agreement with the decrease in Erk1/2 phosphorylation around this time (Figure 3.1). The Y239/240 site may have functions other than Grb2 recruitment, which may be regulated by differential phosphorylation. This could explain the fact that this site remained phosphorylated for longer despite Erk1/2 phosphorylation being downregulated following prolonged EGF stimulation. In any case, mechanisms such as Sos phosphorylation will prevent further activation of Ras despite the possibility that Grb2 can remain bound to this site on Shc up until 60 minutes of EGF stimulation.

NGF stimulation of PC12 cells led to a much lower level of Shc phosphorylation on tyrosines 239/240 than EGF stimulation (Figure 3.2A upper and middle panels). The p52 isoform was the predominant tyrosine phosphorylated isoform in cells exposed to NGF, but upon longer exposure of the X-ray film, phosphorylation of the p66 (between ten and 60 minutes) and the p46 (all stimulated time points) isoforms could also be detected (data not shown). The onset of phosphorylation is slightly slower than after EGF stimulation and the maximum is reached after ten minutes exposure of cells. By 120 minutes the levels had decreased slightly, but were still comparable with that of ten minutes, which indicates that the phosphorylation of Shc is not downregulated following prolonged exposure to NGF. Y317 was also phosphorylated in cells exposed to NGF (Figure 3.2B). The levels of pY317 did not seem to change throughout the time course, or if anything, increase slightly, which again is in accordance with prolonged Erk1/2 activation. The levels of pY317 in Figure 3.3B cannot be directly compared between NGF, EGF and FGF2 stimulation, as a longer exposure of the western blot was required to observe phosphorylation in response to NGF and FGF2 stimulation. Interestingly, p66 Shc was not phosphorylated on Y317 following NGF stimulation, which is in agreement with its involvement in other cellular functions such as apoptosis, that are not necessarily activated by RTKs [231, 232].

Exposure of PC12 cells to FGF2 resulted in yet another pattern of Shc phosphorylation. Whereas TrkA recruits both Shc and FRS2 and these two docking proteins compete for the same binding site on the receptor [70], the FGFR recruits mainly FRS2. The function of Shc in FGFR signalling has not been clearly elucidated, although it has been shown to be phosphorylated in response to FGF2 stimulation [100, 103, 104]. Following exposure to FGF2, Shc was slightly more phosphorylated on Y239/Y240 compared to unstimulated cells (Figure 3.2A). However the amount of pY239/pY240 Shc in FGF2-stimulated cells was significantly less than in EGF and NGF stimulated cells, which is in agreement with its minor role as a Grb2 recruiting adaptor in FGFR signalling [47]. Y317 was only phosphorylated to very low levels, if at all, in response to FGF2 stimulation. Only a longer exposure of the X-ray film revealed any bands for pY317 Shc, and the levels were not significantly greater than basal levels (Figure 3.2B). The p46 isoform seems to slightly more phosphorylated on Y317 in stimulated than in unstimulated cells. However, the levels of phosphorylation are also low and the importance of this observation is unclear.

Clear differences in the tyrosine phosphorylation pattern of the two Shc Grb2-binding sites after exposure of PC12 cells to NGF, EGF or FGF2 were observed, and these were in agreement with the differential levels of Erk1/2 phosphorylation patterns observed (Figure 3.1B). Thus although all three receptors recruit the same proteins, there is a high level of specificity in terms of phosphorylation threshold and duration. These factors are likely to contribute to the generation of highly controlled downstream signals.

3.2.3 Differences in FRS2 phosphorylation downstream of all three growth factor receptors

FRS2 exhibits decreased electrophoretic mobility upon cell stimulation with EGF or FGF. This characteristic is due to serine/threonine phosphorylation of FRS2, and Erk1/2 and MEK have been show to play a role in this process [55]. A similar gel shift was detectable in PC12 cells stimulated with the three different growth factors (Figure 3.3A). In unstimulated cells, FRS2 appeared as a single band of approximately 65kDa, whereas stimulation with EGF, NGF or FGF resulted in a gel shift. This reduced electrophoretic mobility is due to serine/threonine phosphorylation as described by Wu *et al.* [55] and results in FRS2 appearing as a band of approximately 90kDa on a western blot (Figure 3.3.A). There was no detectable difference in the FRS2 mobility shift induced by NGF compared to EGF stimulation. In both cases the majority of FRS2 showed retarded electrophoretic mobility and appeared as the higher band of approximately 90kDa in all stimulated time points. In unstimulated cells, FRS2 appeared as a band of approximately 65kDa which is indicative of its unmodified state. Only a small amount of FRS2 remained as the 65kDa form in EGF and NGF stimulated cells. The gel shift does not indicate changes in tyrosine phosphorylation, which needs to be addressed separately to assess the involvement of FRS2 in Grb2 recruitment downstream of the different receptors (Figure 3.3B).

In contrast a larger amount of FRS2 still appeared as the lower molecular weight form in FGF2 stimulated cells, indicating that this had not yet undergone serine/threonine phosphorylation. This observation may indicate a greater involvement of FRS2 in signalling from the FGFR at all time points, since downregulation via negative feedback does not occur to the same extent observed following NGF or EGF stimulation. Additionally, the shift to the higher molecular weight band occurs slightly later than after EGF or NGF stimulation (Figure 3.3A: two versus five minutes). The differences

in FRS2 gel shift certainly indicate a different temporal regulation of the involvement of this docking protein downstream of the different receptors. Altogether, the gel shift occured immediately after Erk1/2 activation and is independent of the level of FRS2 tyrosine phosphorylation (Figure 3.3B). Thus the gel shift may not necessarily reflect the involvement of FRS2 in signalling complex formation and positive receptor signalling. The serine/threonine phosphorylation may simply act as a mechanism to prevent excessive tyrosine phosphorylation and is important in regulating the type and intensity of signals initiated from various RTKs [55, 110].

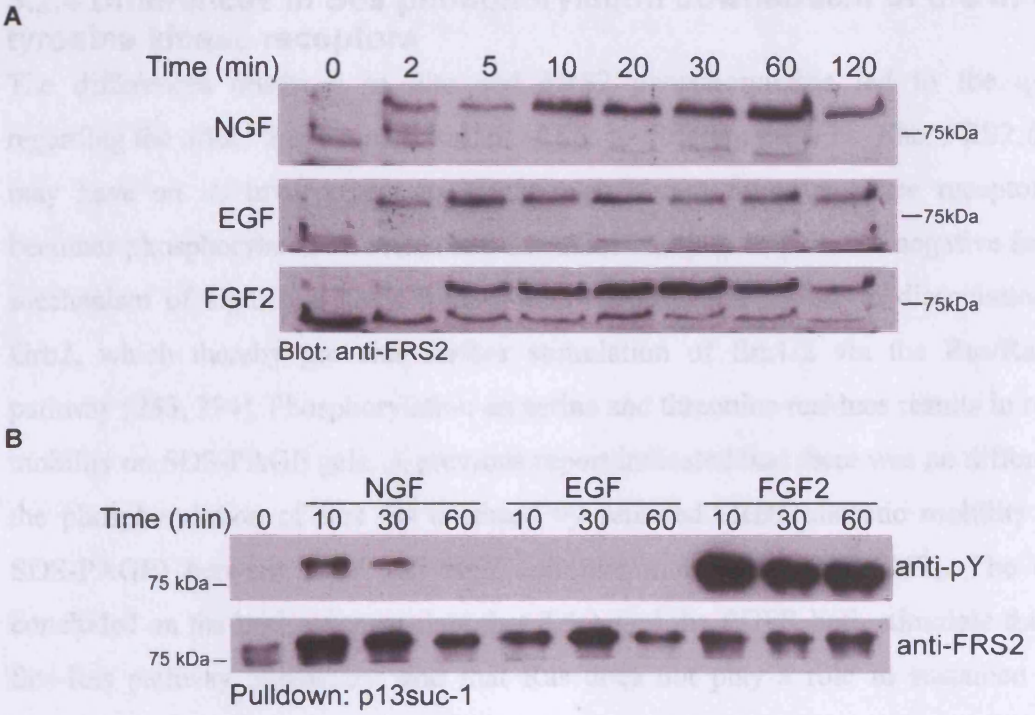


Figure 3.3: Gel shift and tyrosine phosphorylation of FRS2 in reponse to exposure of cells to NGF, EGF or FGF2 PC12 cells were serum-starved overnight and stimulated with 20ng/ml of NGF, EGF or FGF2. 200μg of whole cell lysate were subjected to SDS-PAGE and immunoblotting using an anti-FRS2 antibody (A). 3mg of whole cell lysate were incubated with GST-p13^{suc-1} on glutathione beads overnight and precipitants were subjected to SDS-PAGE and immunoblotting with an anti-phosphotyrosine antibody (B). The results are representative of two independent experiments.

The serine/threonine phosphorylation of FRS2 did not directly affect its tyrosine phosphorylation (Figure 3.3B). Precipitation of FRS2 using a GST-p13^{suc-1} fusion protein revealed that despite its gel shift, FRS2 (only the 90kDa form) was heavily tyrosine phosphorylated following FGF2 stimulation (Figure 3.3B). Although the level of tyrosine phosphorylation declined slightly by 60 minutes of exposure to FGF2, the level remained high even throughout this prolonged stimulation period. EGF stimulation

did not result in tyrosine phosphorylation, whereas NGF only resulted in a fairly low level of FRS2 tyrosine phosphorylation that declined by 30 minutes (Figure 3.3B: lane 3). Following 60 minutes of NGF stimulation, FRS2 tyrosine phosphorylation had virtually disappeared, which seems to be in contrast with the prolonged activation of the Erk1/2 pathway. Even if FRS2 does not recruit Grb2 during later time points of TrkA activation, it would be expected to remain tyrosine phosphorylated to retain the ability to recruit other signalling proteins such as Shp2 and Crk.

3.2.4 Differences in Sos phosphorylation downstream of the three tyrosine kinase receptors

The differences observed in Shc and FRS2 phosphorylation led to the question regarding the effect that the recruitment of Sos to different proteins (Shc, FRS2, EGFR) may have on its involvement in signal transduction from the three receptors. Sos becomes phosphorylated on serine and threonine residues as part of a negative feedback mechanism of signalling from RTKs. This modification results in dissociation from Grb2, which thereby prevents further stimulation of Erk1/2 via the Ras/Raf/MEK pathway [233, 234]. Phosphorylation on serine and threonine residues results in retarded mobility on SDS-PAGE gels. A previous report indicated that there was no difference in the phosphorylation of Sos (as assessed by retarded electrophoretic mobility during SDS-PAGE) between EGF and NGF stimulation of PC12 cells [67]. The authors concluded on the basis of these data that TrkA and the EGFR both stimulate the Grb2-Sos-Ras pathway transiently, and that Ras does not play a role in sustained Erk1/2 phosphorylation. The report indicated that sustained Erk1/2 activation in response to NGF stimulation was due to prolonged Rap1 activation via Crk and C3G. Since the study did not include the effect of FGF stimulation on Sos serine/threonine phosphorylation, it was chosen to partially repeat the experiment. The effect of all three growth factors on Sos electrophoretic mobility (i.e. gel shift) as a result of this phosphorylation was assessed.

Subtle differences in the gel shift of Sos were observed when comparing stimulation of PC12 cells with all three growth factors (Figure 3.4). Upon EGF stimulation, Sos portrays retarded mobility on the gel between five and 30 minutes, which indicates that within a short period, a large proportion of Sos became serine/threonine phosphorylated and therefore unavailable for recruitment to the plasma membrane via Grb2. This would

provide a mechanism to ensure transient Erk1/2 activation. Interestingly, a decrease in total cellular level of Sos was observed upon prolonged exposure of cells to EGF (Figure 3.4: middle panel, levels of Sos compared to PLCγ loading control). This may provide an additional mechanism to avoid prolonged activation of Erk1/2 during continuous exposure to EGF.

In NGF stimulated cells, a mobility shift of Sos was observed only after ten minutes, which is in agreement with the slightly delayed onset of Erk1/2 phosphorylation (Figure 3.1). However, degradation of Sos was only observed after prolonged exposure of cells to NGF and this occurred at a later time point and to a lesser extent than after EGF stimulation (Figure 3.4: 60-120 minutes upper and lower panel versus 20-120 minutes middle panel). This indicates that although the overall pattern of Sos phosphorylation is fairly similar to that of EGF, the association with Grb2 does not need to be prevented by degradation of Sos. Other mechanisms may regulate the association of these two proteins in cells stimulated with different growth factors.

It was further interesting to note differences in the electrophoretic mobility of Sos between NGF and FGF2 stimulated cells. Both of these growth factors induce prolonged Erk1/2 activation. However, the gel shift observed after FGF2 stimulation was less compared to NGF (or EGF) stimulation and was only observed after ten minutes. NGF stimulation leads to prolonged activation of Erk1/2 by recruitment of the guanine nucleotide exchange factor C3G, which results in Rap1 activation. This makes activation of Ras via Sos redundant. The data presented here indicate the possibility that Grb2 and Sos remain associated for prolonged periods of time following FGF2 stimulation due to a lower level of Sos phosphorylation via negative feedback. Subsequently, FGF2 stimulation of PC12 cells might result in prolonged activation of Ras via the recruitment of the Grb2/Sos complex to the activated receptor.

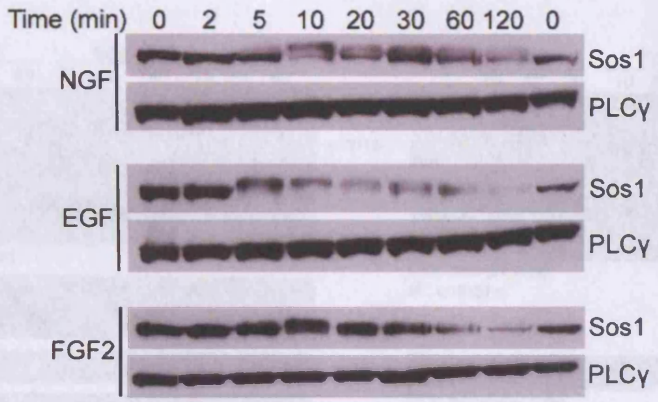


Figure 3.4: Sos gel shift in response to stimulation with NGF, EGF or FGF2 Lysates (200µg) from PC12 cells stimulated with 20ng/ml of each NGF, EGF or FGF2 for various time periods after overnight serum-starvation were subjected to SDS-PAGE. All gels were run under the same conditions at the same time. Western blots were probed with an anti-Sos antibody and an anti-PLCγ antibody as a loading control. The results are representative of three independent experiments.

3.2.5 Immunoprecipitation and pulldown experiments confirm the recruitment of adaptor proteins into different early signalling complexes

To assess whether the adaptor proteins Shc, FRS2, Grb2 and Sos were not only post-translationally modified differentially upon stimulation of PC12 cells with NGF, EGF or FGF2 but were also found in distinct early signalling complexes downstream of each growth factor receptor, immunoprecipitation and pulldown experiments were carried out (Figure 3.5).

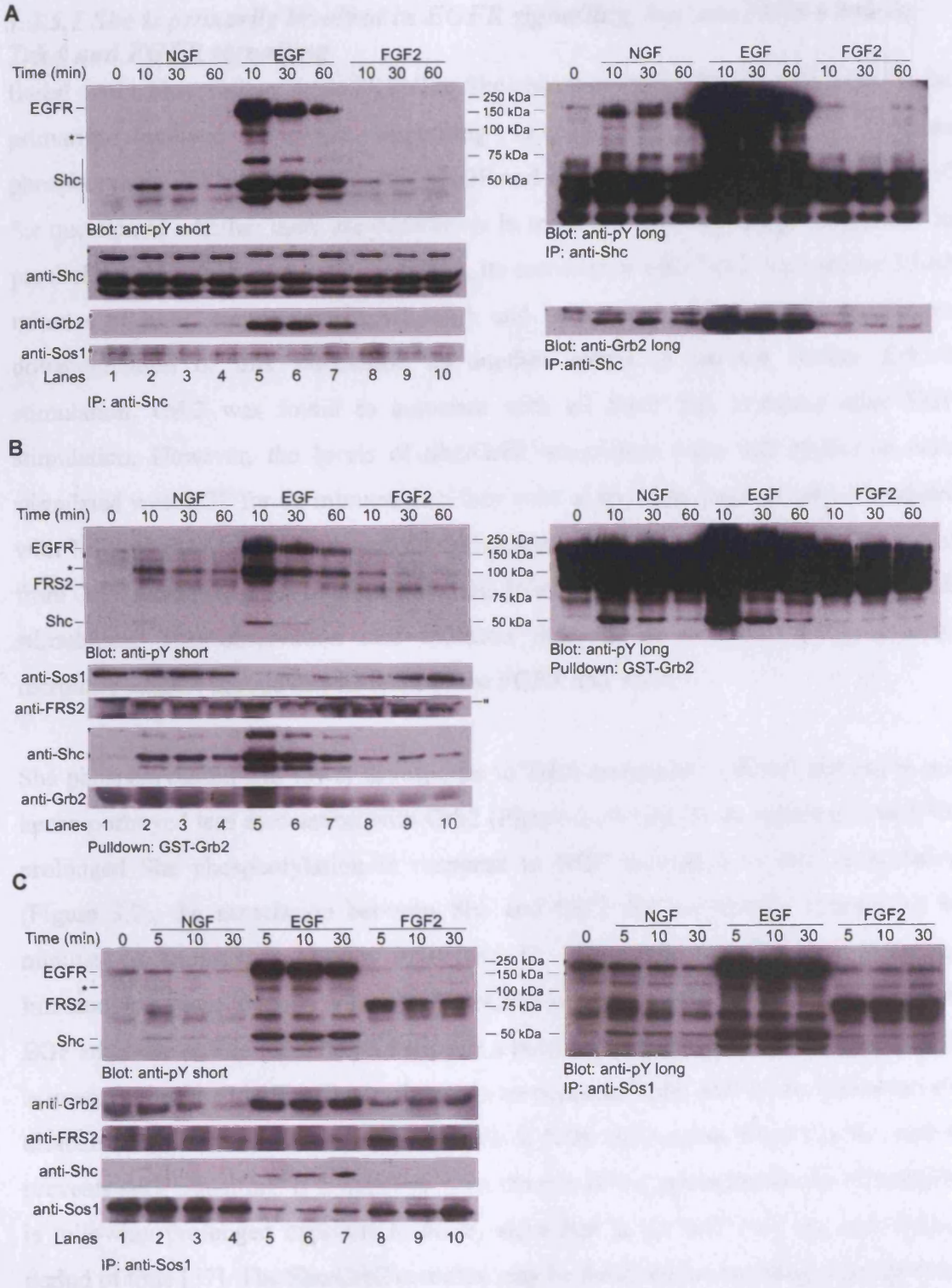


Figure 3.5: Formation of different multiprotein complexes in response to stimulation with NGF, EGF or FGF2 PC12 cells were stimulated with 20ng/ml of the respective growth factors for ten, 30 or 60 minutes and lysed. Whole cell lysates were subjected to immunoprecipitation with an anti-Shc antibody (A), an anti-Sos1 antibody (C) or a pulldown using Grb2-GST (B). Western blots were initially probed with an anti-phosphotyrosine antibody, stripped and re-probed with various antibodies against Sos, Shc, Grb2, or FRS2. Longer exposures of the western blot are shown on the right. The label for the EGFR applies to EGF-stimulated lanes only. * phosphoprotein likely to be Gab1, " non-specific band recognized by anti-FRS2 antibody

3.2.5.1 Shc is primarily involved in EGFR signalling, but also plays a role in TrkA and FGFR signalling

Based on the observations made regarding Shc phosphorylation, this protein seems to be primarily involved in EGF signalling (Figure 3.2). Nonetheless, tyrosine phosphorylation of both sites following NGF and FGF stimulation of PC12 cells raised the question of whether there are differences in recruitment to signalling complexes. In parallel with the dephosphorylation of Shc, its association with Grb2 declined by 30-60 minutes of EGF stimulation (Figure 3.5A and B: lanes 6 and 7), which implicates downregulation of this association as another means to prevent further Erk1/2 stimulation. Grb2 was found to associate with all three Shc isoforms after EGF stimulation. However, the levels of Shc/Grb2 association were still higher in cells stimulated with EGF for 60 minutes than they were at any time point in cells stimulated with NGF or FGF2. This indicates that other mechanisms such as dissociation of Sos from Grb2 must take place to allow downregulation of Erk1/2 following prolonged EGF stimulation. This observation also indicates that Shc is not the only/main Grb2 recruiting adaptor protein downstream of the FGFR and TrkA.

Shc phosphorylation was lower in response to TrkA compared to EGFR activation and hence portrayed less association with Grb2 (Figure 3.5A and B). In agreement with the prolonged Shc phosphorylation in response to NGF compared to EGF stimulation (Figure 3.2), the association between Shc and Grb2 did not change throughout 60 minutes of stimulation (Figure 3.5B and C: lanes 2-4, anti-Shc and anti-Grb2 immunoblots respectively). Although less Grb2 was associated with Shc compared to EGF stimulation, this observation indicates a potential role for Shc in prolonged Erk1/2 activation via the Grb2/Sos/Ras pathway in response to TrkA activation. However, the dissociation of Grb2 and Sos by 60 minutes of NGF stimulation (Figure 3.5C: lane 4) prevents such signalling. It is not clear what the role of the persistent Shc/Grb2 complex is following prolonged exposure to NGF, since Ras is not activated for a prolonged period of time [67]. The Shc/Grb2 complex may be involved in recruiting other proteins such as Gab1 to the TrkA receptor that are important in activation of additional downstream pathways.

In response to FGF2, Shc was not heavily phosphorylated (Figures 3.2 and 3.5A), but it bound to Grb2 throughout the 60 minute stimulation period (Figure 3.5A and B: lanes 8-10 and longer exposures). Neither Shc phosphorylation nor the amount of Grb2

associated with it changed throughout the 60 minutes of stimulation, although both were elevated compared to unstimulated cells (Figure 3.5A and B: lanes 8-10 versus lane 1). This indicates a lack of downregulation of this interaction and confirms that indeed the recruitment of Grb2 via Shc may play a minor role in prolonged Erk1/2 activation downstream of the FGFR. More Shc seemed to be precipitated by GST-Grb2 (Figure 3.5B) than Grb2 was co-precipitated with Shc (Figure 3.5A). This observation may be explained by the fact that an excess of GST-Grb2 was added to the cell lysates, which can interact with a greater pool of phosphorylated Shc than would be the case intracellulary.

3.2.5.2 The Grb2-Sos complex is regulated differentially by all three receptors

Grb2 has been shown to be constitutively associated with Sos, although this interaction can be enhanced upon activation of certain tyrosine kinase receptors and association of Grb2 with Shc [235]. The recruitment of Sos to the membrane via Grb2 leads to activation of the Ras-Raf-MEK pathway. Since subtle differences in Sos gel shift were observed (Figure 3.4), the changes in its interaction with Grb2 were analysed using immunoprecipitation and pulldown techniques (Figure 3.5B and C). Although the Sos gel shift occurred within a short period of stimulation (five to ten minutes) of all three growth factors, the association pattern with Grb2 did not change remarkably throughout 60 minutes. This is surprising in light of the fact that it has been previously reported that the gel shift is due to serine/threonine phosphorylation which then leads to dissociation of Grb2 [233]. Perhaps a large enough pool of Sos remains unphosphorylated and therefore able to bind Grb2, or additional mechanisms are important in regulating dissociation of these two proteins.

Grb2 and Sos were indeed constitutively associated in PC12 cells (Figure 3.5B and C: lane 1). Interestingly only EGF stimulation resulted in a true increase in the amount of Grb2 associated with Sos (Figure 3.5A). Unfortunately in the Grb2-GST pulldown, the actual amount of Sos precipitated with Grb2 could not be determined, because Sos runs at the same molecular weight as the EGF receptor, and the large amount of EGFR co-precipitated with Grb2 masked the Sos antibody recognition site and hampered its detection (Figure 3.5B: lanes 5-7). However, immunoprecipitation using an anti-Sos antibody revealed that the amount of Grb2 associated with Sos did not significantly decrease even after 30 minutes of EGF stimulation (Figure 3.5C: lanes 5-7), which indicates that this interaction can persist despite serine/threonine phosphorylation of Sos

(Figure 3.4). It is likely that the decreased interaction of Grb2 with Shc and the EGFR after 60 minutes of stimulation (Figure 3.5A and B) results in relocation of the Grb2/Sos complexes away from the membrane, which means that Ras activation can no longer take place. This is in agreement with previous findings, which indicated that Sos phosphorylation in response to EGF stimulation resulted in dissociation of the Grb2/Sos complex from Shc and the EGFR rather than Sos dissociation from Grb2 [236-238]. Co-localisation analysis of these two proteins in complex would be important to elucidate more clearly how downregulation occurs and whether re-localisation to a different compartment (i.e. removal of Sos from the plasma membrane and hence the membrane-anchored Ras) plays a role in this process.

In comparison to EGF stimulation, the amount of Grb2 associated with Sos declined with prolonged NGF stimulation (Figure 3.5C), which may be consistent with a switch from the Sos/Ras/Raf pathway for Erk1/2 activation to the C3G/Rap1/Raf pathway as previously reported [67]. Whereas Sos immunoprecipitation indicated loss of Grb2/Sos interaction by 30 minutes of NGF stimulation, the Grb2 pulldown suggested prolonged association until 60 minutes (Figure 3.5B and C). However, the latter is likely an effect caused by the presence of excess GST-Grb2 that is able to bind the maximum amount of Sos present and therefore does not necessarily represent the true level of association between these two proteins. This is further supported by the fact that no increase in Sos/Grb2 association was observed from basal in the Grb2 pulldown experiment (Figure 3.5B: lane 1 versus lanes 2-10). It is not clear why dissociation occurs after prolonged NGF stimulation but not after prolonged EGF stimulation, although similar gel shift patterns of Sos were observed. However, this indicates that the existence of different signalling complexes is able to promote the correct, desired signals in response to different growth factors and is able to control downregulation of the signal in different ways. It has been proposed previously that the recruitment of different kinases to signalling complexes downstream of various receptors and their phosphorylation of specific sites on Sos may affect the stability of Grb2/Sos complexes [238]. Further studies would need to be undertaken to assess whether differences in the sites on which Sos becomes phosphorylated by different kinases are important in mediating differential dissociation from Grb2.

Perhaps the most striking observation was, that in contrast to NGF stimulation, the Grb2/Sos interaction was slightly increased with prolonged exposure of cells to FGF

(Figure 3.5B and C: lanes 8-9 versus lane 10). This is in agreement with the less prominent Sos gel shift observed in FGF-stimulated cells (Figure 3.4). Since both NGF and FGF initiate very similar cellular responses (i.e. prolonged Erk1/2 activation and subsequent differentiation), one might have expected the FGFR and TrkA to initiate activation of similar signalling pathways. However, the differential regulation of the dissociation of the Grb2/Sos complex indicates that this is not the case. Prolonged activation of TrkA leads to Erk1/2 phosphorylation via activation of Rap1 by recruitment of Crk and C3G while the Grb2/Sos/Ras signalling pathway is only important for transient Erk1/2 activation [67]. The data presented in this chapter indicate that sustained FGFR activation does not cause downregulation of the Grb2/Sos signalling pathway and continues to signal via these proteins for a prolonged period of time.

3.2.5.3 FRS2 is the major component recruiting the Grb2/Sos complex downstream of the FGFR

FRS2 has been described as the main component recruiting Grb2 to the FGFR. As described in section 3.2.5.1, Shc may play a minor role in Grb2 recruitment downstream of the activated FGFR. However, Shc phosphorylation (and hence association with Grb2) is much lower than that following activation of the EGFR, which indicates that Shc does not carry out a major role in Erk1/2 activation by the FGFR. FRS2 was found to be the major tyrosine phosphorylated protein in FGF stimulated cells (Figures 3.3B and 3.5B and C). Its association with Grb2 and Sos decreased slightly by 60 minutes of FGF stimulation (Figure 3.5B and C: lane 10 versus lanes 8-9), which is concomitant with its slight dephosphorylation (Figure 3.3B: lane 10). This dephosphorylation of FRS2 is in contrast with the persistent or even somewhat increased association between Grb2 and Sos (section 3.2.6.2) observed at later time points of FGF exposure. However, FRS2 remains highly phosphorylated throughout prolonged FGF stimulation, so that sufficient bindings sites for Grb2 are retained, which would allow Grb2 and Sos to remain associated near the plasma membrane and lead to Ras activation. The level of FRS2 phosphorylation at later time points may therefore be sufficient for recruitment of enough Grb2/Sos complexes to maintain Erk1/2 phosphorylation. Altogether, FRS2 remained phosphorylated more strongly and for a longer period of time than in cells stimulated with NGF and the interaction with Grb2 and Sos was more pronounced in FGF2 stimulated cells. This indicates that although both of these growth factors cause differentiation by inducing prolonged Erk1/2 phosphorylation, the formation of

signalling complexes differs downstream of TrkA and FGFR. The differential recruitment of adaptor proteins to the activated receptors regulates the activation of downstream targets by diverse but convergent signalling pathways.

Virtually no FRS2 co-precipitated with Grb2 and Sos in EGF stimulated cells (Figure 3.5B and C: lanes 5-6), which is in accordance with previous findings that FRS2 does not associate with Grb2 following EGF stimulation [55]. FRS2 is not a major docking protein downstream of the activated EGFR, since Grb2 can be recruited to the receptor directly or via Shc. FRS2 has been shown to be part of a mechanism for downregulation of signalling from the EGFR [55], which would explain its presence in EGFR signalling complexes at later time points (Figure 3.5B: lane 7). Nonetheless, precipitation of FRS2 with $p13^{suc-1}$ (Figure 3.4B) revealed no significant phosphorylation of FRS2, which is in agreement with the fact that FRS2 is not a major component of EGFR signalling. Even if FRS2 was involved in signal downregulation, it would not be expected to interact with Grb2 and Sos in any case, which further explains the absence of this signalling protein from the EGFR-induced signalling complexes analysed in this study.

Surprisingly, FRS2 was only minimally phosphorylated in response to TrkA compared to FGFR activation (Figure 3.4B). FRS2 seems to only be involved in Grb2/Sos recruitment in the earlier stages of TrkA signalling, since it only co-precipitated with Sos five minutes after NGF stimulation (Figure 3.5C) but was not found in complex with Grb2 at any of the time points used for the pulldown assay (i.e. ten to 60 minutes, Figure 3.5B: lanes 2-4). The GST-$p13^{suc-1}$ pulldown revealed that FRS2 was maximally phosphorylated around ten minutes of NGF stimulation and that it was completely dephosphorylated by 60 minutes (Figure 3.4B). In contrast to FGF stimulation, where phosphorylation and association with Grb2 occurred up until 60 minutes, NGF stimulation did not cause prolonged recruitment of Grb2 and Sos to the activated TrkA receptor via FRS2. This observation seems to indicate that in response to NGF stimulation, Shc is the main Grb2/Sos recruiting adaptor, whereas FRS2 plays an important role in recruitment of other proteins such as Crk into larger multimeric complexes. Recruitment of such proteins may be required to initiate differentiation. It was interesting to note that FRS2 phosphorylation was not maintained throughout a longer time period, and it must therefore be able to carry out some of its roles in the absence of major tyrosine phosphorylation. It could be a possibility that the Grb2 binding sites on FRS2 are dephosphorylated, but that the remaining tyrosine residues

(Y436 or Y471, which are Shp2 binding sites) remain phosphorylated to a small extent. Phosphorylation of tyrosine residue Y436 would allow Crk interaction with FRS2 to be retained [67], but may not be sufficient to allow high levels of FRS2 to be detected by an anti-phosphotyrosine antibody. Since the Grb2/Sos and the Shc/Sos association was also downregulated in cells exposed to NGF for prolonged periods of time (Figure 3.5A and C), other pathways must be activated to results in prolonged Erk1/2 activation. These are likely to act via Rap1 rather than Ras. Altogether, the phosphorylation of Sos, and the signalling complexes it is involved in, differed between all three receptors. This indicates that differential recruitment of proteins like FRS2 may be an important regulatory mechanism of growth factor-specific signalling intracellularly.

3.2.6 Grb2 on its own does not display discrete localisation patterns after NGF, EGF or FGF2 stimulation

To analyse possible changes in the protein recruitment to the different receptors and changes in co-localisation of proteins following NGF, EGF or FGF stimulation, PC12 cells were stably transfected with C-terminally GFP-tagged Grb2. Individual clones expressing different amounts of the construct were isolated using dilution cloning (Figure 3.6A). The cells expressing the lowest amount of Grb2-GFP (clone C2) were selected for confocal imaging, as they were bright enough for confocal microscopic analysis and did not show any breakdown products due to extensive overexpression. The cells were stimulated with NGF, EGF or FGF for various time periods and midsection confocal images of cells were analysed. No clear differences in Grb2 localisation were observed between unstimulated and stimulated cells or between cells stimulated with different growth factors (Figure 3.6B). Most of the Grb2-GFP resided in the cytoplasm, with some localisation to the membrane as well as the nucleus. The ubiquitous expression meant that there was no detectable change in the localisation of Grb2 upon stimulation. Membrane localisation was perhaps somewhat more pronounced in NGF and FGF stimulated cells throughout the later time points (30-60 minutes), but the differences observed were minimal. Consequently co-localisation or ideally FRET/FLIM analysis of Grb2-GFP and its RFP-tagged binding partners Shc, Sos and FRS2 is required to obtain meaningful information regarding differences in spatio-temporal protein recruitment and interaction following EGF, NGF or FGF stimulation. Although this approach was attempted, various experimental problems were encountered. Firstly, analysis by confocal imaging was hampered by fairly high

levels of autofluorescence of PC12 cells in the RFP emission range, which required high levels of expression of the tagged constructs. Secondly, high expression levels could not be achieved, so that co-localisation and FLIM studies could not be carried out. These will need to be addressed in a separate study.

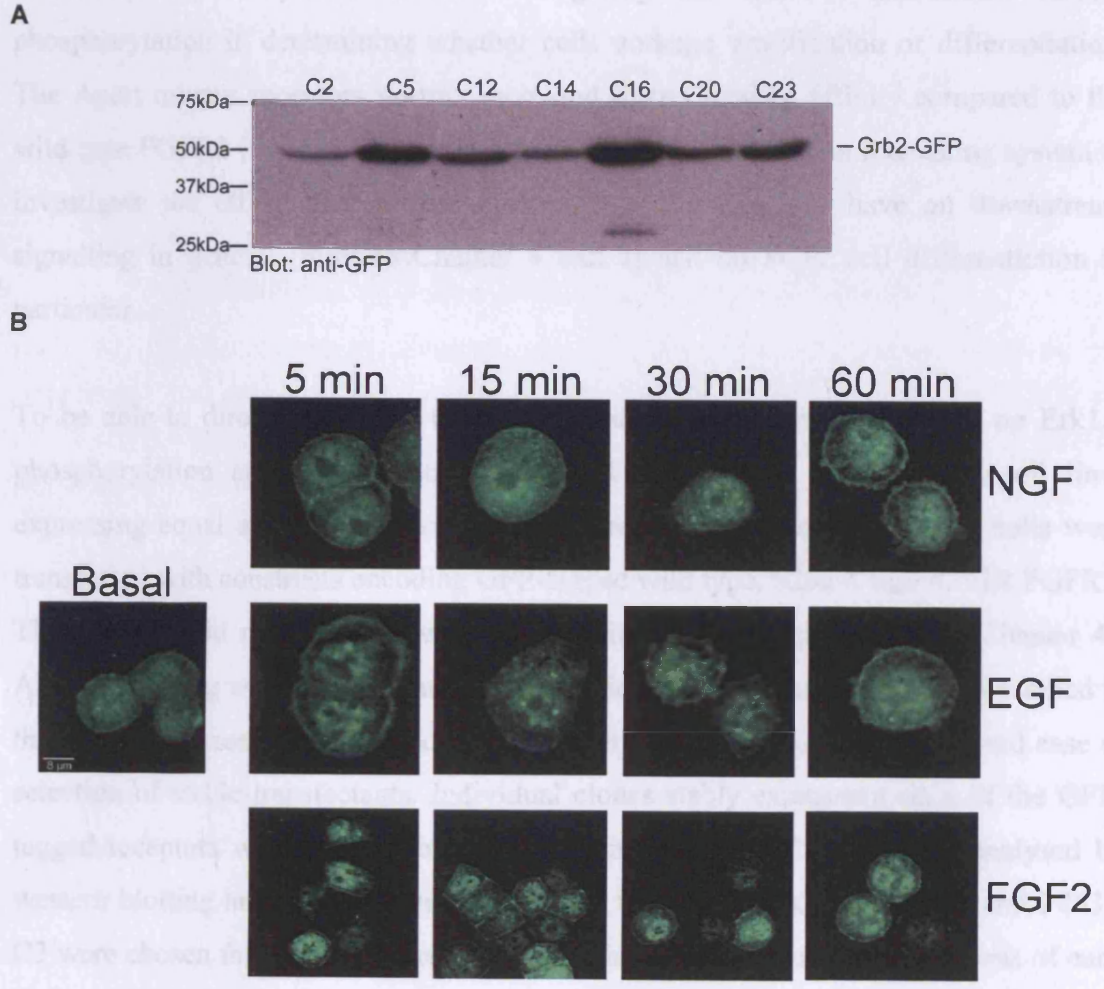


Figure 3.6: Grb2 localisation in response to NGF, EGF or FGF2 stimulation Stable cells expressing Grb2-GFP were created and individual clones were analysed by western blotting (A). The cells (clone C2) were seeded on glass coverslips coated with poly-D-lysine and serum-starved overnight. They were subsequently stimulated with 20ng/ml of each respective growth factor for different periods of time, fixed in 4% paraformaldehyde and mounted on glass slides. Cells stimulated with FGF2 are represented at lower magnification. Images were obtained using a Leica SP2 confocal system and Leica TCS NT control software and are representative of at least ten cells imaged (B).

3.2.7 The threshold of Erk1/2 activation as well as its duration is important for initiation of correct cellular responses to growth factor stimulation

The effects of Apert syndrome mutations on FGFR2 signalling will be the main focus of Chapters 4 and 5. However, it was chosen to use the Apert syndrome mutations as an additional model system whilst investigating the role of differential Erk1/2 phosphorylation in determining whether cells undergo proliferation or differentiation. The Apert mutant receptors portray increased ligand binding affinity compared to the wild type FGFR2 [7, 161]. This property made these mutations an interesting system to investigate the effects that altered receptor/ligand interactions have on downstream signalling in general (refer to Chapter 4 and 5) and on PC12 cell differentiation in particular.

To be able to directly compare the effects that the mutant receptors have on Erk1/2 phosphorylation and differentiation compared to the wild type FGFR2, cell lines expressing equal amounts of each respective receptor were created. PC12 cells were transfected with constructs encoding GFP-tagged wild type, S252W and P253R FGFR2. The GFP-tag did not interfere with functionality of the receptor (refer to Chapter 4). Although the tag was not necessary for the studies carried out in PC12 cells presented in this chapter the same GFP-tagged receptors were used for their availability and ease of selection of stable transfectants. Individual clones stably expressing each of the GFP-tagged receptors were isolated by dilution-cloning in 96-well plates and analysed by western blotting and fluorescence microscopy. Clones WT C6, S252W C2 and P253R C3 were chosen for further experiments since they expressed comparable levels of each of the three receptors (Figure 3.7A). Clonal variation was excluded by assessment of different clones for the same downstream signalling responses to FGF stimulation. Confocal microscopy revealed that the receptors were targeted to the plasma membrane, which indicates that GFP-tagging does not interfere with correct receptor folding or post-translational modification (Figure 3.7B).

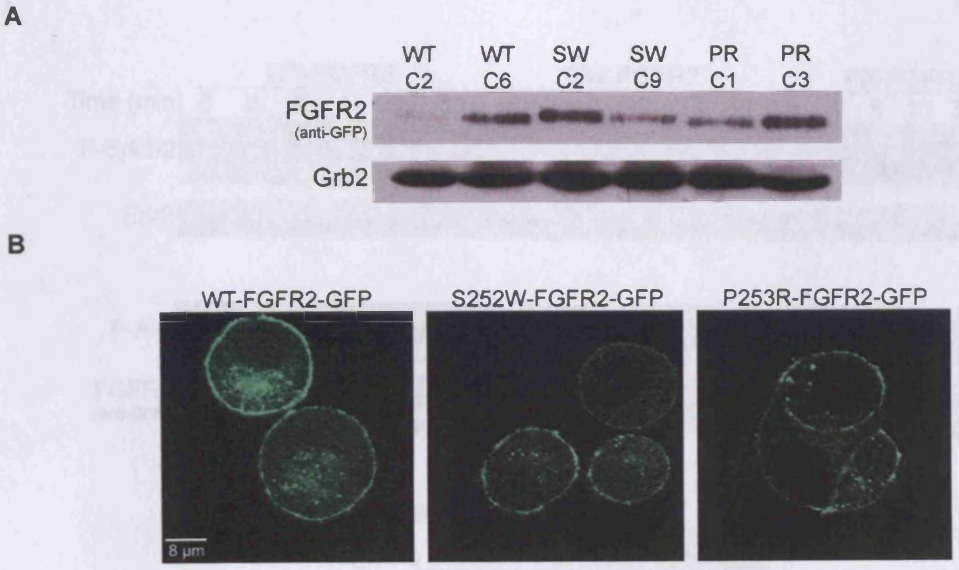


Figure 3.7: Creation of stable PC12 cell lines expressing the wild type, S252W and P253R FGFR2 respectively PC12 cells were stably transfected with constructs encoding the wild type, S252W and P253R FGFR2-GFP and individual clones were obtained by dilution cloning and subsequently analysed by western blotting with an anti-GFP-antibody (A) and by confocal microscopy (B).

To assess the effects of the Apert syndrome mutations on signalling pathways downstream of the FGFR2, cells expressing the wild type, S252W or P253R FGFR2 were stimulated with FGF9 for various periods of time and the levels of Erk1/2 phosphorylation were assessed by western blotting (Figure 3.8A and B). Erk1/2 activation in response to FGF9 stimulation was greatly enhanced in cells expressing either the S252W or the P253R compared to the wild type receptor. PI3K signalling has been implicated with a role in neurite outgrowth, but is primarily important for cell survival in response to NGF stimulation [239, 240]. Nonetheless, changes in the activation of this kinase might contribute to changes in the differentiation pattern of PC12 cells expressing the Apert mutant receptors compared to the wild type FGFR2. To investigate whether the Apert mutations affect the activation of PI3K, the levels of Akt phosphorylation were assessed by western blotting, as a read-out of PI3K activity. No changes in the levels of Akt phosphorylation were observed, which indicates that differential activation of this pathway does not contribute to any alterations in formation or stability of neurites in cells expressing either of the mutant receptors (Figure 3.8A). Untransfected PC12 cells did not portray Erk1/2 activation since they do not express any FGFR2 (Figure 3.8A) [100], which indicates that all effects observed are due to activation of the exogenously expressed receptors and not activation of endogenous FGFRs.

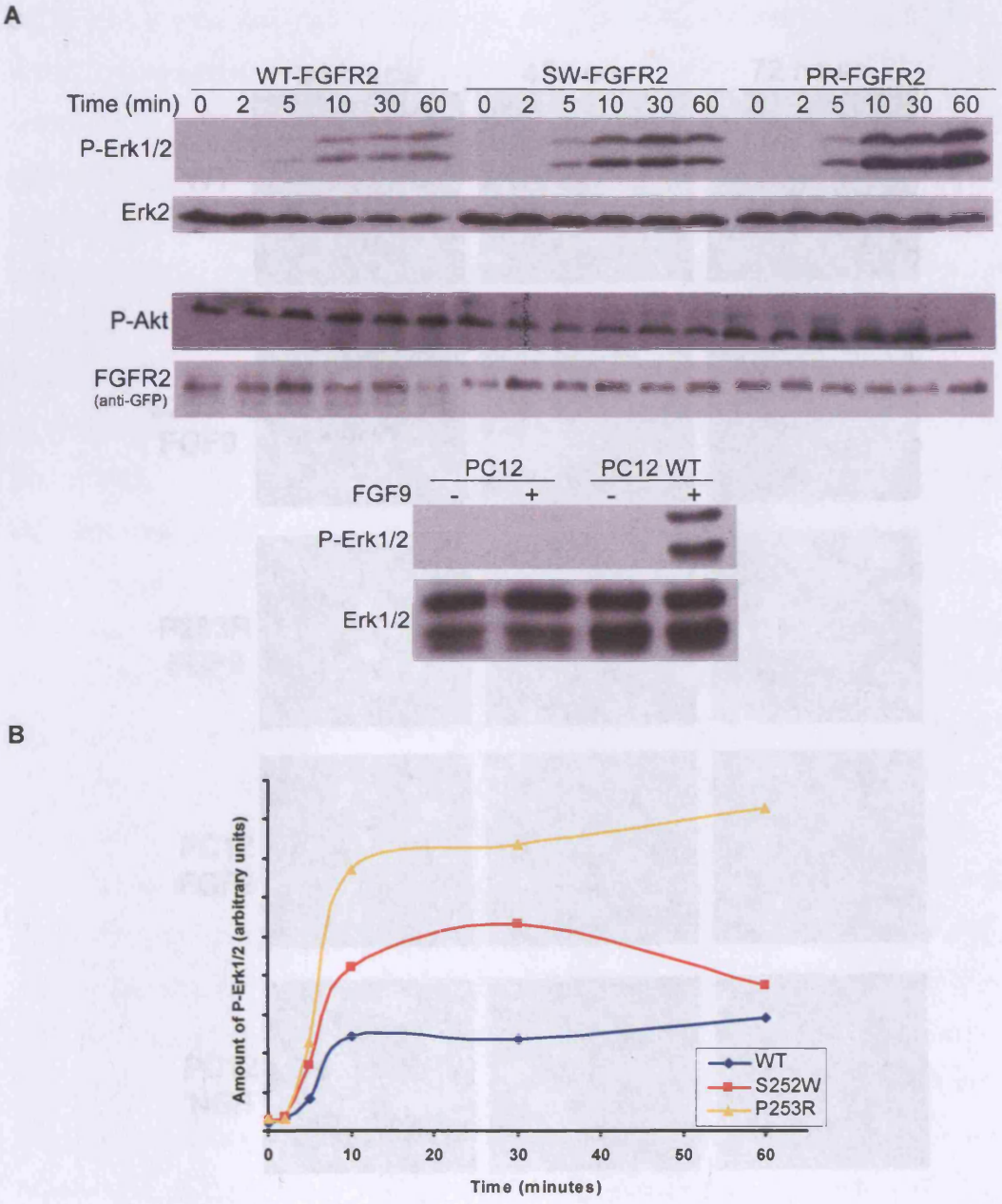
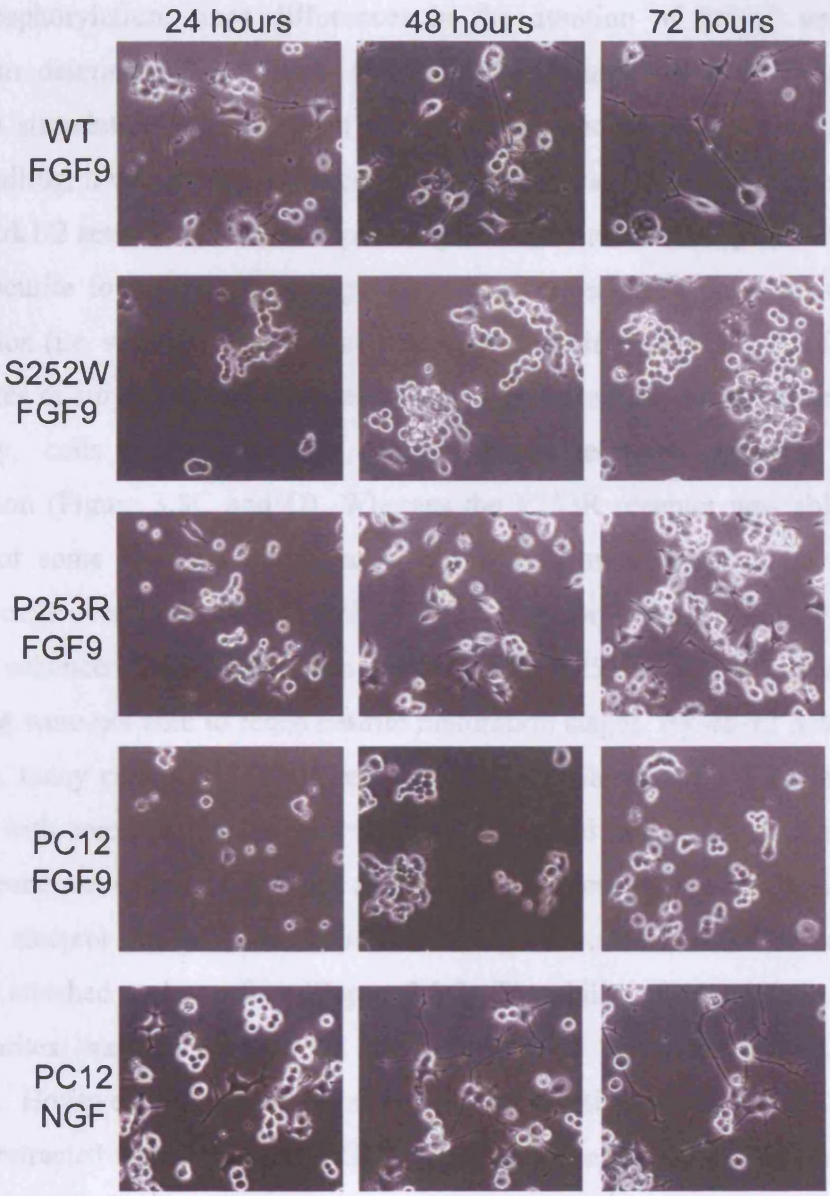
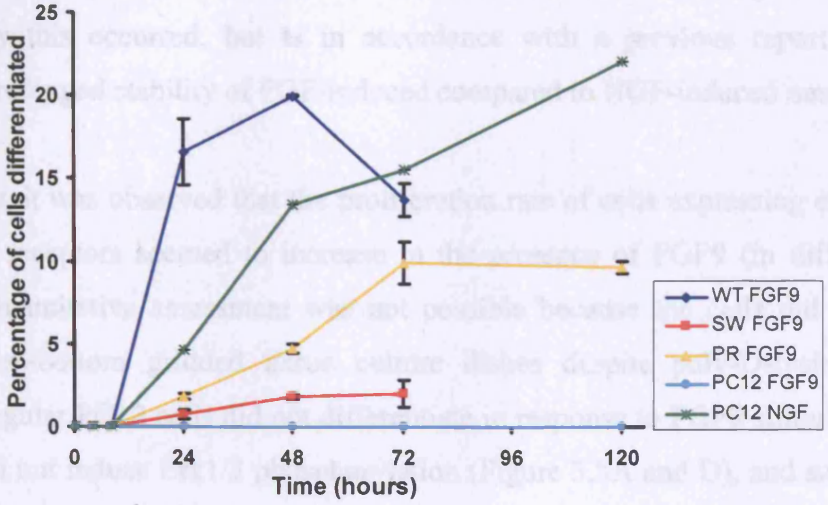


Figure 3.8: Effect of Apert syndrome mutations on Erk1/2 phosphorylation and differentiation in PC12 cells PC12 cells stably transfected with constructs encoding the wild type, S252W and P253R FGFR2-GFP were plated on poly-D-lysine coated dishes, serum-starved overnight and stimulated with 20ng/ml FGF9 for various periods of time. Western blots were probed with antibodies against phospho-Erk1/2, phospho-Akt (a prolonged exposure of the blot is presented), GFP and stripped and re-probed with an anti-Erk1/2 antibody (A). The amount of phosphorylated Erk1/2 was quantified using densitometry and represented graphically (B). For differentiation assays cells were plated on poly-D-lysine coated coverslips and incubated with 20ng/ml FGF9 or 100ng/ml NGF for 72 hours. Cells (400-500 per sample) in random fields of view were photographed and scored for neurites greater than twice the cell body diameter at the times indicated (C and D, page 116). The data are representative of two independent experiments with the exception of NGF stimulation of untransfected PC12 cells.

C


24 hours
48 hours
72 hours
WT FGF9
S252W FGF9
P253R FGF9
PC12 FGF9
PC12 NGF

D


Percentage of cells differentiated
25
20
15
10
5
0
0
24
48
72
96
120
Time (hours)
WT FGF9
SW FGF9
PR FGF9
PC12 FGF9
PC12 NGF

PC12 cells are an ideal model system to investigate the cellular effects of changes in Erk1/2 phosphorylation, since differences in the duration of Erk1/2 activation are important in determining cell fate. Although the duration of Erk1/2 activation in response to stimulation of the mutant receptors was not altered compared to normal FGFR signalling, it was greatly enhanced (Figure 3.8A and B). It was proposed that this enhanced Erk1/2 activation in cells expressing the Apert mutant receptors would lead to enhanced neurite formation. This might have been revealed in the form of (i) faster differentiation (i.e. within a shorter time period after addition of FGF), (ii) formation of more neurites or (iii) elongated neurites in cells expressing the Apert mutant receptors. Surprisingly, cells expressing the Apert mutant receptors showed diminished differentiation (Figure 3.8C and D). Whereas the P253R receptor was able to induce formation of some neurites, barely any neurite outgrowth was evident in S252W-expressing cells even after 72 hours of FGF9 stimulation (Figure 3.8D). Furthermore, despite the enhanced Erk1/2 activation caused by the P253R receptor, cells expressing this receptor were not able to reach neurite maturation stages. By 48-72 hours of FGF9 stimulation, many cells were in the early stages of differentiation (i.e. flattened, less round and with some membrane protrusions), but could not progress to form proper extended neurites even after 120 hours of FGF9 stimulation (Figure 3.8C). Activation of the S252W receptor did not even induce flattening of the cells, which remained round and loosely attached to the surface (Figure 3.8C). The ability of the wild type FGFR2 to induce neurites was comparable to the response of untransfected cells to NGF stimulation. However, the neurites induced by stimulation of the FGFR2 were less stable and retracted with prolonged FGF9 stimulation (beginning at 72 hours but more pronounced at 120 hours). The cells detached from the poly-D-lysine coated surface, which is why no cell counts could be undertaken at 120 hours (Figure 3.8D). It is unclear why this occurred, but is in accordance with a previous report indicating decreased prolonged stability of FGF-induced compared to NGF-induced neurites [241].

Additionally, it was observed that the proliferation rate of cells expressing either of the two mutant receptors seemed to increase in the presence of FGF9 (in differentiation medium). Quantitative assessment was not possible because the cells did not adhere well to glass-bottom gridded tissue culture dishes despite poly-D-lysine coating. However, regular PC12 cells did not differentiate in response to FGF9 stimulation since this FGF did not induce Erk1/2 phosphorylation (Figure 3.8A and D), and subsequently most cells died in the differentiation medium (which lacks serum for cell growth) by 72

hours. On the contrary, cells expressing the S252W or the P253R receptors did not die or detach and displayed increased numbers in differentiation medium. The fact that these cells seemed to proliferate in medium that was lacking serum but contained FGF9 indicated that somehow the enhanced Erk1/2 activation caused the opposite response normally expected to take place in response to prolonged activation of this pathway.

3.3 Discussion

3.3.1 Duration and magnitude of signals have to be regulated to achieve specific cellular responses

Previous findings indicated that prolonged Erk1/2 activation downstream of the FGFR and TrkA leads to differentiation, whereas EGFR-induced transient Erk1/2 activation leads to proliferation. Comparison of the signal duration in response to NGF, EGF and FGF confirmed and enhanced these results. Interestingly, it was found using the Apert syndrome FGFR2 that precise quantitative regulation of Erk1/2 activation is required to achieve specificity in the cellular outcome. The activation of either of the two Apert syndrome mutant FGFR2s resulted in prolonged but also enhanced Erk1/2 activation in comparison to the wild type receptor. Surprisingly, this did not induce differentiation. This is interesting in light of the fact that the duration of Erk1/2 activation is believed to be the most important regulator of the proliferation/differentiation decision in PC12 cells. The observation that the threshold and not only the duration of Erk1/2 activation is important to achieve the correct cellular response sheds new light on the way in which the regulation of cell signalling is viewed. The fact that the Apert mutations can alter cell fate by changing the level of activation of this kinase rather than the length of time for which it is activated, indicates that cell regulation is highly controlled and several aspects have to be tightly controlled to achieve the correct downstream response.

As described in later chapters (refer to Chapters 4 and 5), the Apert syndrome mutations not only affect Erk1/2 signalling, but also result in other effects on both the receptor and downstream signalling events. Hence the possibility that the changes in differentiation pattern observed in PC12 cells expressing the mutant receptors are not solely due to the increased activation of the Erk1/2 kinase cannot strictly be excluded. Interestingly, the Apert syndrome mutations did not result in any changes in the phosphorylation of Akt (Figure 3.7B), which is an indication that alteration of the PI3K pathway is not responsible for the changes in differentiation. Expression of constitutively active MEK1 or Ras is sufficient for induction of neurite outgrowth and PI3K signalling is more important for cell survival than neurite outgrowth [240, 242, 243]. This supports the fact that the PI3K pathway does not play a role in causing the altered differentiation pattern observed in the presence of the Apert syndrome mutations. Nonetheless, further work investigating the changes that cause enhanced Erk1/2 activation (such as altered

assembly of signalling complexes involving Grb2, Sos, FRS2, Shc and other adaptor proteins) would be extremely interesting and valuable in gaining insight into the mechanism by which differentiation is prevented by the S252W and P253R receptors despite prolonged Erk1/2 activation. Although activation of the Erk1/2 pathway was deemed sufficient for PC12 cell differentiation [243], other studies have indicated the requirement for the activation of additional pathways involving for example Src or PLCγ for this process [244]. Analysis of the effects of the Apert mutations on these pathways has to be undertaken to exclude the possibility that it is not the altered Erk1/2 signal strength but the changes in other pathways that result in lack of differentiation in cells expressing the Apert syndrome FGFR2.

The observation that both the duration and the magnitude of activation of a certain pathway are important for specific downstream responses adds further complexity to the regulation of signal transduction. Tight control of the strength/magnitude (i.e. the number of downstream proteins such as Erk1/2 that are activated) or the duration of a signal can be achieved by the formation of signalling complexes. Multiprotein complex formation is important for modification of signal duration, since downregulation may be prevented or enhanced, by for example sheltering from dephosphorylation or by specifically recruiting proteins involved in signal downregulation such as phosphatases or ubiquitin ligases, respectively. At the same time, assembly of proteins into a complex may also provide a means to specifically regulate the magnitude (e.g. level of phosphorylation or activation) of a signal generated. Such regulation could be achieved by recruitment of proteins into slightly different complexes downstream of various receptors. Depending on the complex formed, both the duration and the magnitude of a signal could be precisely regulated. This is important for allowing activation of specific cellular responses, such as differentiation or proliferation, by the same signalling pathways (in this case Erk1/2). If quantitative differences such as signal duration and magnitude were not present and could not be tightly controlled, all receptors in a given cell type might lead to the same cellular response, because they often employ the same proteins and activated the same downstream pathways (refer to Figures 1.2, 1.3 and 1.4).

3.3.2 Recruitment of unique signalling complexes to different RTKs regulates downstream signal transduction

3.3.2.1 Receptor-specific differences regulate protein recruitment

Since the discovery that various cellular responses to different growth factors are regulated by activation of very similar intracellular pathways including the Erk1/2, PLCγ and PI3K pathways, it has been shown that spatio-temporal regulation of factors such as protein (re)location and signal duration are important mediators of biological specificity (reviewed in [145]). The work presented in this chapter has shown that differential regulation of the same downstream pathway can be regulated by assembly of the same proteins into unique signalling complexes downstream of different RTKs.

The recruitment of the proteins Shc, FRS2, Grb2 and Sos into multiprotein complexes was found to be intrinsically different between the EGFR, FGFR and TrkA. Regulation of the assembly of different complexes is mediated by various factors such as the availability of binding sites for adaptor proteins on the receptor, differential levels of phosphorylation and exclusion of certain proteins from complexes by occupation of binding sites by other interacting partners. Firstly, each of the three receptors possesses different binding sites for various adaptor proteins, which affects the recruitment of proteins into multimolecular assemblies (Figure 3.9). Secondly, the stoichiometry with which individual sites on the receptors are phosphorylated also affects the recruitment of adaptor proteins and whether or not certain protein-protein interactions can take place. Highly regulated and very precise phosphorylation of the FGFR1 has been shown to occur [15]. Undoubtedly this plays an important role in regulating the recruitment of specific proteins to the receptor at different times, not only by providing binding sites for selected proteins, but by also adding temporal control over which pathways are activated and in which order. Thirdly, it has been shown that the cytoplasmic region of the EGFR undergoes various structural changes upon stimulation by EGF, since certain antibody epitopes are only unmasked in the phosphorylated but not the unphosphorylated EGFR [245]. Such mechanisms may add additional control over the availability of binding sites on receptors. Altogether the availability of binding sites for different adaptor proteins on the individual receptors is an important determinant of the type of signalling complex that will be assembled.

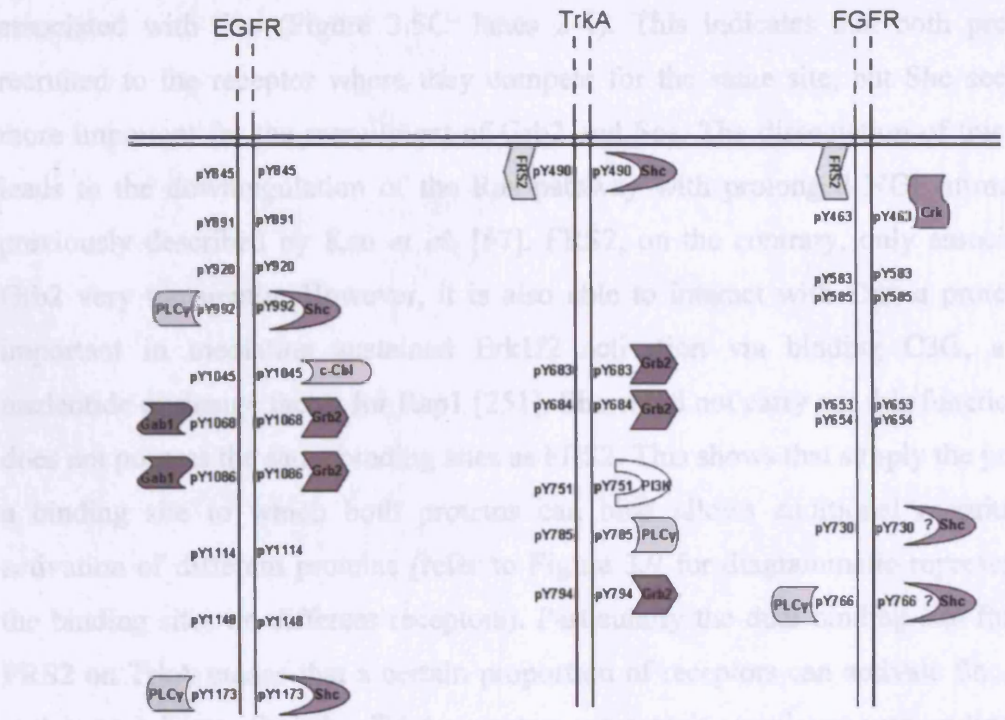


Figure 3.9 Diagrammatic representation of the known binding sites for signalling proteins on the EGFR, FGFR and TrkA The known phosphorylation sites in the intracellular regions of the three different receptors as well as the signalling proteins that have been shown to bind to these sites are indicated. Y683 and Y684 on TrkA and Y653 and Y654 on FGFR are activation loop tyrosines. Shc has been suggested to bind to Y730 and Y766 of the FGFR1, but it is unclear whether this interaction occurs in vivo. FRS2 binds to the FGFR1 in a phosphotyrosine-independent fashion in the juxtamembrane region. Only the main binding partners of TrkA are shown. Shc and FRS2 compete for the same PTB domain binding site surrounding phosphorylated Y490 of TrkA. FRS2 has been suggested to bind to the EGFR in a single report, but no site has been identified and this finding is unconfirmed by other investigations. The binding sites for the FGFR are for a generalised model and do not represent a specific FGFR isoform. Adapted from [33, 48, 68-70, 246-250].

3.3.2.2 Differential recruitment of FRS2 and Shc to the EGFR, FGFR and TrkA is an important regulator of transient versus prolonged Erk1/2 activation

The differential recruitment and involvement of FRS2 presents a clear example of how the presence of different binding sites on the receptor intrinsically regulates formation of receptor-specific complexes (refer to Figure 3.10 for diagrammatic representation). TrkA possesses a shared recognition and binding site for the Shc and FRS2 PTB domains (Figure 3.9), which was reflected in the fact that both Shc and FRS2 were phosphorylated and involved in Grb2/Sos recruitment (Figure 3.5). Whereas Shc was able to bind to Grb2 for prolonged periods of time with a slight decline in association with prolonged exposure to NGF (Figure 3.5A and B), FRS2 was hardly detectable in complex with Grb2 at all, and only at the earliest time points (Figure 3.5B and C). The amount of FRS2 in complex with Sos declined faster compared to the amounts

associated with Shc (Figure 3.5C: lanes 2-4). This indicates that both proteins are recruited to the receptor where they compete for the same site, but Shc seems to be more important for the recruitment of Grb2 and Sos. The dissociation of this complex leads to the downregulation of the Ras pathway with prolonged NGF stimulation as previously described by Kao *et al.* [67]. FRS2, on the contrary, only associates with Grb2 very transiently. However, it is also able to interact with Crk, a protein that is important in mediating sustained Erk1/2 activation via binding C3G, a guanine nucleotide exchange factor for Rap1 [251]. Shc could not carry out this function since it does not possess the same binding sites as FRS2. This shows that simply the presence of a binding site to which both proteins can bind allows additional recruitment and activation of different proteins (refer to Figure 3.9 for diagrammatic representation of the binding sites on different receptors). Particularly the dual binding site for Shc and FRS2 on TrkA means that a certain proportion of receptors can activate Shc-mediated pathways, whereas the other TrkA receptors can recruit complexes surrounding FRS2 or differences in binding of each protein after various periods of stimulation may tightly regulate the transient and prolonged activation of different pathways.

Although interaction of the PTB domain of FRS2 with the EGFR has been documented in a single report [55], no specific FRS2 binding site on the EGFR has been described to date. The lack of FRS2 tyrosine phosphorylation in response to EGFR activation also indicates that FRS2 does not seem to play a major role in terms of Grb2 recruitment. The multitude of proteins that are recruited to TrkA compared to the EGFR may form the basis for initiation of differentiation rather than proliferation. Since the EGFR lacks the ability to activate for example FRS2-mediated pathways, it may not be able to induce prolonged Erk1/2 activation nor other pathways that are required for sustained neurite outgrowth (refer to Figures 3.9 and 3.10). Thus indeed the presence of different binding sites is important for regulation of protein recruitment and activation downstream of different RTKs.

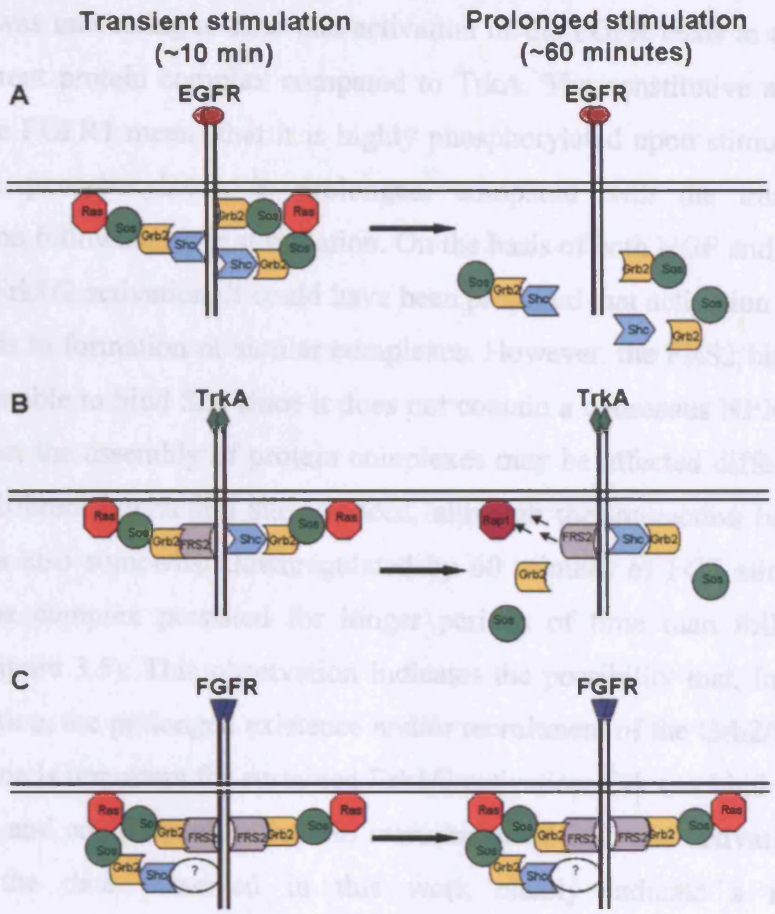


Figure 3.10: Diagrammatic representation of the signalling complexes assembled around each RTK and the changes they undergo with prolonged receptor activation The role of Shc (light blue), Grb2 (yellow), Sos (green), FRS2 (grey), Ras (red), Rap1 (pink) in EGFR, TrkA and FGFR signalling based on the results presented in this chapter and previous studies is represented diagrammatically. Other proteins that are also recruited to these receptors and play a role in generation of specific signals have been omitted for simplicity.

(A) Transient activation of the EGFR leads to recruitment of the Grb2/Sos complex directly and indirectly via Shc, FRS2 is not involved in Grb2 recruitment to this receptor. Recruitment of Sos leads to activation of Erk1/2 via Ras. Prolonged exposure to EGF results in EGFR signal downregulation by dissociation of the Grb2/Sos complex from the EGFR and Shc, as well as dissociation of Sos from Grb2 and the Shc/Grb2 complex. This would prevent sustained activation of Erk1/2 [233].

(B) The Grb2/Sos complex is recruited to TrkA via Shc and FRS2 following transient activation of this receptor. Following prolonged TrkA activation by NGF results in pronounced dissociation of Sos from Grb2 as well as dissociation of the FRS2/Grb2 and Shc/Grb2 complexes. Prolonged Erk1/2 activation must therefore be achieved via activation of Rap1 via recruitment of Crk and C3G to FRS2 [67].

(C) Transient activation of the FGFR leads primarily to recruitment of the Grb2/Sos complex to FRS2. Shc may play a minor role in Grb2/Sos recruitment, and is itself recruited to the FGFR via unknown mechanisms such as interaction with one or more unidentified proteins (as indicated by the question mark). Prolonged exposure to FGF does not result in downregulation of the Grb2/Sos/Ras pathway as seen following prolonged NGF stimulation, but the FRS2/Grb2/Sos and Shc/Grb2/Sos complexes persist and would be expected to be able to induce prolonged Erk1/2 activation via Ras [17].

Moreover, it was interesting to note that activation of the FGFR leads to the formation of yet a different protein complex compared to TrkA. The constitutive association of FRS2 with the FGFR1 means that it is highly phosphorylated upon stimulation of this receptor. The phosphorylation is prolonged, compared with the transient FRS2 phosphorylation following NGF stimulation. On the basis of both NGF and FGF leading to prolonged Erk1/2 activation, it could have been proposed that activation of the FGFR and TrkA leads to formation of similar complexes. However, the FRS2 binding site on the FGFR is unable to bind Shc since it does not contain a consensus NPXY motif [4]. This means that the assembly of protein complexes may be affected differently by the presence of different interaction sites. Indeed, although the interaction between Grb2 and FRS2 was also somewhat downregulated by 60 minutes of FGF stimulation, the FRS2/Grb2/Sos complex persisted for longer periods of time than following NGF stimulation (Figure 3.5). This observation indicates the possibility that, in response to FGFR stimulation, the prolonged existence and/or recruitment of the Grb2/Sos complex to the membrane is important for sustained Erk1/2 activation. Crk can bind to the FGFR directly [122], and could therefore lead to recruitment of C3G and activation of Rap1. Nonetheless, the data presented in this work clearly indicate a role of the FRS2/Grb2/Sos complex in prolonged Erk1/2 activation in response to FGF2 stimulation. The hypothesis that differences in the formation of the Grb2/Sos complex and prolonged interaction with FRS2 is largely responsible for the prolonged Erk1/2 activation in response to FGF stimulation was further supported by both Sos and FRS2 being less serine/threonine phosphorylated than following EGF or NGF stimulation (Figures 3.3 and 3.4). This is indicative of a lesser degree of downregulation of this pathway following FGFR activation, which contributes to the prolonged phosphorylation of the Erk1/2 pathway. Hence the presence or absence of specific binding sites for FRS2 on the various receptors is an important regulator of the assembly of very precise yet different multiprotein complexes as well as the ultimate downstream signal initiated.

In addition to the differences in FRS2 recruitment to and involvement in signalling from the different receptors, Shc was also found to be differentially involved downstream of all three receptors (refer to Figure 3.10 for diagrammatic representation). Shc can bind the EGFR via both its PTB and its SH2 domain, whereas it interacts with TrkA via the PTB domain only [49, 50, 69, 184]. Direct association between the FGFR and Shc has not been reported to date, although co-precipitation of Shc with the FGFR3 and FGFR1

has been shown [105, 106]. However, Shc can be phosphorylated in response to EGF and FGF even in the absence of any phosphorylation sites on their receptors [51, 103], which indicates that interaction with the receptor is not required for phosphorylation and therefore its involvement in signalling pathways. This means that regulation of the way in which Shc is involved in signalling from a certain RTK has to be achieved in a different way than simply by availability of binding sites on a receptor. If Shc can be phosphorylated in the absence of binding to a receptor directly (i.e. membrane recruitment is sufficient for it to be phosphorylated as long as a receptor tyrosine kinase is active), theoretically all three receptors should be able to phosphorylate Shc equally. It is therefore remarkable to note that clear differences in Shc phosphorylation by each receptor existed (Figure 3.5). Whereas Shc was strongly phosphorylated on both sites (Y239/Y240 and Y317) in response to EGF stimulation, both NGF and FGF stimulation only caused mild phosphorylation (with that induced by NGF being somewhat stronger). This observation portrays the differences in the relative involvement of Shc in Grb2 recruitment in response to the different growth factors. It is not clear how these differences are achieved and certainly the availability of binding sites on the receptors enhances Shc phosphorylation (i.e. it can bind to the EGFR, but has to compete with FRS2 for the binding site on TrkA and most likely does not interact with the FGFR). Nonetheless other factors such as varied access to the vicinity of the receptor kinase due to assembly of different signalling complexes or different abilities of the individual kinases to phosphorylate the tyrosine residues on Shc might also play an important role in differential Shc phosphorylation and subsequent involvement in signal transduction.

3.3.2.3 Temporal regulation of the formation and dissociation of different complexes by the EGFR, FGFR and TrkA

The finding that FGFR signalling seems to lead to sustained Erk1/2 activation by prolonged Ras activation via Grb2/Sos recruitment is interesting in light of recent studies highlighting the 'switch' from Ras to Rap1 signalling in transient versus sustained Erk1/2 activation following NGF stimulation [67, 228]. In these studies it was proposed that both transient activation of Ras and Rap1 was similar in response to EGF or NGF stimulation, but that prolonged Erk1/2 activation in response to NGF occurred primarily via the activation of Rap1 via C3G instead of Ras activation via Sos. Recruitment of C3G via Crk was shown to be mediated via FRS2 [67]. Although the data presented in this chapter indicate that different mechanisms lead to prolonged Erk1/2 activation in response to FGF stimulation, further investigation of the activation

of Ras and Rap1 in response to this growth factor would be required. It would be very interesting, if differences in the way in which NGF and FGF lead to prolonged Erk1/2 activation would be confirmed. Differences in how these two growth factors achieve the same cellular outcome indicate that intrinsic differences in signalling pathways occur and that even the same cellular outcome (i.e. sustained Erk1/2 activation) can be achieved via different mechanisms. Based on the results presented in this study, it seems possible that the FGFR leads to prolonged phosphorylation of Erk1/2 purely by activation of Ras, since the FRS2/Grb2/Sos complex existed for prolonged periods of FGF stimulation. However, since Crk has been shown to associate with the FGFR directly [122], the possibility that Rap1 is activated in addition to prolonged Ras activation via FRS2/Grb2/Sos cannot be excluded. Since slight differences exist in the stability of neurites induced by FGF and NGF [241], it would be particularly interesting to investigate whether these are the result of differences in signalling complexes formed upon NGF or FGF stimulation, which lead to differences in Ras and Rap1 activation as well as variations in activation of secondary signalling pathways.

3.3.2.4 Importance of complex formation in the activation of diverse secondary pathways

In addition to leading to differences in Erk1/2 activation, the importance of assembling different protein complexes downstream of various receptors lies in the fact that they may differentially activate signalling pathways other than Erk1/2. The activation of other, secondary pathways from different signalling complexes is required to activate those cellular responses that are not shared between different RTKs. Differential involvement of Shc and FRS2 is not only important in activation of the Erk1/2 pathway. Their presence in different signalling complexes in response to EGF, NGF or FGF stimulation is also a key regulator of the activation of secondary pathways, which may be important in regulation of the overall cellular response.

For example, recruitment of Gab1 to the FRS2/Grb2 complex is an important regulator of activation of the PI3K pathway. This means that depending on the level of FRS2 phosphorylation and the type of complex it forms with other proteins downstream of different receptors numerous convergent as well as diverse signalling pathways can be activated. The assembly of protein complexes is important in this process, because linear pathways would not be able to result in such fine-tuned differences.

In addition to different pathways activated by the differential recruitment of FRS2, Shc may also be involved in the activation of secondary pathways beyond Erk1/2. Recent studies have implicated Shc with roles other than Grb2 recruitment. This property may be important when considering the role of Shc in TrkA and FGFR signalling. Downstream of these receptors it may play a minor role in Erk1/2 activation when compared to EGFR signalling but may be important in assembly of a signalling complex that activates secondary pathways important for differentiation. The differential phosphorylation of the two major sites on Shc may be important for this process. Phosphorylation of the Y239/Y240 site was more stimulation-dependent downstream of TrkA and the FGFR than Y317, although Y317 has been reported to be the main Grb2 binding site [198]. Since NGF and FGF both do not cause a great increase in phosphorylation of the Y317 site, this may indicate differences in Shc roles compared to EGF stimulation, which causes both sites to be strongly phosphorylated. The importance of the various sites in terms of Erk1/2 activation and recruitment of proteins such as Grb2 to the activated receptors would have to be investigated separately to quantitatively assess the different contribution they may make to signal transduction downstream of the different receptors.

3.3.3 Intrinsic differences in RTK signalling versus cell-specific interpretation of the same general signal

This work has shown that both quantitative (different duration of activation) and qualitative (different protein recruitment patterns and complex formation) traits are intrinsic properties to each receptor system analysed. Generally such intrinsic diversity forms the basis for the precise regulation required to achieve signalling specificity in response to different growth factors. Disturbance of these intrinsic properties may result in the wrong downstream response, as demonstrated by the Apert syndrome mutations in the FGFR2. The observation that both the duration and strength of Erk1/2 phosphorylation are essential for generating the correct cellular signal (namely differentiation instead of proliferation) indicates that the signal initiated by each receptor is not simply a 'go' signal that is interpreted differently by cells of different origin (as proposed Simon in [252]). It is important for the regulation of downstream responses such as differentiation, which receptor activates the signal (in this case Erk1/2). Although each receptor can activate similar pathways, assembly of unique

signalling complexes allows the generation of individual signals from different receptors.

The model in which basically all RTKs just provide a single signal that activates specific responses has been supported by findings that the PDGFR and FGFR both induce transcription of very similar early genes in NIH 3T3 cells [253]. Such a mechanism may be important in the context of the whole organism. Nonethless, in contrast the data presented herein based on the PC12 model system provide evidence that intrinsic differences in signals from various RTKs are present. This diversity is important for precise cellular regulation of signal transduction and is mediated by assembly of different complexes downstream of each receptor. Nonetheless, PC12 cells are a tissue culture model system, which may not necessarily represent the events taking place inside a whole organism. Although some intrinsic differences in signalling from various RTKs exist, the alteration of receptor and ligand expression levels during development of an organism may also play a very important role in signal regulation. In the organismal context, the availability of ligand may also play a great role in regulation of specific signals. In such a context where receptor and ligand expression are tightly regulated, the different receptors may indeed primarily create a 'go' or 'on' signal for mainly the same signalling pathways [252]. In the cell culture model, such differences are difficult to control and may therefore be overlooked in terms of their importance in regulation of signal specificity. Nonetheless, the results presented highlight the fact that precise regulation of downstream signalling pathways can be achieved by assembling precise, unique multiprotein complexes downstream of different RTKs.

In conclusion, the data presented in this chapter provided further evidence for the existence of intrinsic differences in RTK signalling and the regulation of these by recruitment of the same proteins into different protein complexes. The formation of specific complexes is important for the regulation of downstream signalling pathways. However, it was also shown that tight control of the 'normal' signalling pathways is required, because changes in the duration and strength/magnitude (as observed for the Apert syndrome mutant FGFR2s) of the signal initiated can affect the cellular responses to receptor stimulation. Thus altogether, the highly fine-tuned differences in protein recruitment downstream of various RTKs provides a means to initiate specific signals without the need to utilise a completely different set of proteins downstream of each receptor.

Chapter 4

Effects of the Apert syndrome mutations on the FGFR2

4.1 Introduction

To date the majority of studies regarding intracellular signal transduction from the fibroblast growth factor receptor (FGFR) have been carried out using the FGFR1 as a model system. Implications that FGFR2, 3 and 4 initiate similar signalling pathways have been made, although differences have been shown to exist between the different FGFR subfamilies [100-102, 254]. In addition, all families of this receptor subclass show very different tissue expression, which indicates the physiological necessity to be able to initiate similar yet distinct signalling pathways in various tissues at different times. Although some studies have indicated subtle differences between the various FGFR isoforms, no detailed investigations of the FGFR2 and the intracellular signalling pathways initiated from it have been undertaken to date.

More importantly, in addition to a lack of in-depth studies of signal transduction from the FGFR2, the effects of the Apert syndrome mutations on early intracellular signalling from this receptor have not been investigated. The effect of the two main mutations (S252W and P253R) on interaction of the extracellular domain of FGFR2 with various FGF ligands in vitro and in vivo have been analysed [160, 161, 163]. Additionally, data have been published highlighting the effects on physiologically relevant cellular outcomes such as differentiation, apoptosis and calcification of bone precursor cell lines, which have given a certain level of insight into the events that lead to manifestation of Apert syndrome [164, 165, 167, 168, 170]. However, depending on the level of maturation of the osteoblasts used, conflicting results were obtained (as described in [164]). Moreover, these findings were not able to provide any detailed information on the effect that the Apert mutations have on the FGFR2 itself or early signalling events emanating from it. Altogether, this makes the Apert syndrome mutations an attractive model system to investigate the effects that mutations in the extracellular region of a receptor may have on spatial and temporal regulation of downstream signalling and the recruitment of adaptor proteins to the activated receptor.

In light of the need for studies focussing on the intracellular signalling events from the FGFR2 and mutants thereof, it was chosen to investigate the effects of the two main Apert syndrome mutations, S252W and P253R, on the FGFR2 itself as well as the recruitment of proteins to its early signalling complexes. The data presented in this chapter highlight the vast number of effects incurred on the FGFR2 by the two Apert

syndrome mutations. The effects of the Apert syndrome mutations on receptor phosphorylation, glycosylation, and ligand binding were analysed. Altogether the results presented demonstrate that the Apert syndrome mutations give rise to more complex effects on the FGFR2 than the previously reported increased ligand binding and altered ligand binding specificity. These various effects may act in conjunction to contribute to altered intracellular signalling from the FGFR2 and hence may play an important role in the manifestation of Apert syndrome.

4.2 Results

4.2.1 Creation of stable cell lines expressing GFP-tagged wild type, S252W or P253R FGFR2

To study the effects of the two main Apert syndrome mutations on FGFR2 signalling, HEK 293T cells were transfected with constructs encoding each of the three receptor forms tagged with enhanced GFP (WT-FGFR2-GFP, S252W-FGFR2-GFP and P253R-FGFR2-GFP, from hereon referred to as the wild type, S252W and P253R receptors). To investigate the effects of the Apert syndrome mutations on the FGFR2 and signal transduction from this receptor a cellular model system that is completely unresponsive to FGF stimulation would by ideal. Fairly widely used systems to study FGFR signalling without any background signalling are the L6 myoblast and BaF3 cell lines, which do not express any endogenous FGFRs [255, 256]. However, the difficulty of achieving high transfection efficiencies and the possible lack of components of FGFR signalling as a result of complete absence of FGFRs made these cells experimentally unfavourable systems. Instead, HEK 293T cells were chosen for their ease of transfection and ability to express exogenous proteins to a high level.

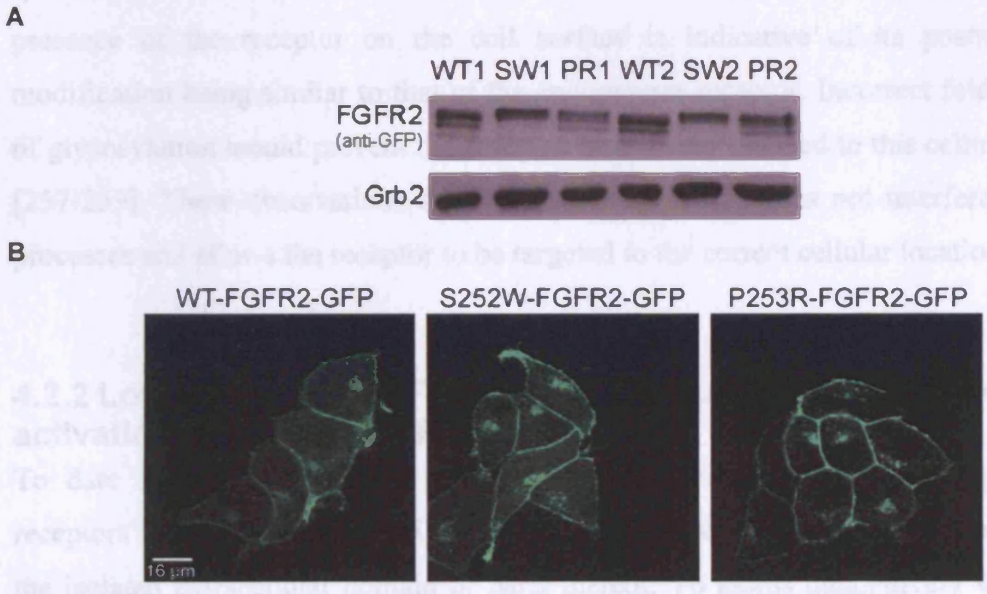


Figure 4.1: Creation of stable HEK 293T cell lines expressing GFP-tagged wild type, S252W and P253R FGFR2 HEK 293T were stably transfected with each of the three FGFR2-GFP constructs and individual clones were isolated by dilution cloning. Equal amounts of whole cell lysate were subjected to SDS-PAGE and immunoblotting with an anti-GFP antibody to probe for similar expression levels (A). Individual clones were analysed by confocal microscopy to assess cellular targeting of the GFP-tagged receptor after seeding on glass slides in growth medium and fixation in 4% paraformaldehyde (B).

To ensure that any differences observed between the three different receptors are solely due to the mutation in the extracellular domain and not different expression levels in pools of stable cells, individual clones expressing each respective receptor construct were created. This was achieved by dilution cloning in 96-well plates after initial selection of stable cell pools. Several clones expressing each receptor construct were isolated and preliminary FGF stimulation experiments confirmed that differences observed between wild type and mutant receptors were not due to clonal variation. Cell lines expressing comparable levels of each receptor were chosen for all subsequent experiments (Figure 4.1A). Western blotting of two independent sets of lysates with an anti-GFP antibody (Figure 4.1A) and visual analysis by fluorescent microscopy revealed that the expression levels of all three clonal cell lines were similar.

Confocal microscopy was carried out to confirm that the GFP-tagged receptors were targeted to the correct cellular compartment, namely the plasma membrane (Figure 4.1B). The GFP-tagged receptors were found to localise mainly at the plasma membrane, with some presence in the perinuclear region. The latter regions most likely represent the endoplasmic reticulum and the Golgi apparatus in which the receptor is synthesised and which therefore contain a lot of GFP-tagged protein (Figure 4.1B). The presence of the receptor on the cell surface is indicative of its posttranslational modification being similar to that of the endogenous receptor. Incorrect folding or lack of glycosylation would prevent the receptor from being targeted to this cellular location [257-259]. These observations confirm that the GFP-tag does not interfere with such processes and allows the receptor to be targeted to the correct cellular location.

4.2.2 Lower doses of FGF are required to cause maximal Erk1/2 activation by the mutant FGFR2

To date several studies have indicated an increased affinity of the Apert mutant receptors for FGF ligand [7, 161]. However, these studies have been carried out using the isolated extracellular domain or parts thereof. To assess qualitatively whether this increased affinity could be observed in vivo, cells expressing the wild type or either of the two Apert mutant receptors were stimulated with increasing concentrations of FGF2 and FGF9 and the effect on the phosphorylation of Erk1/2 (a major target activated downstream of the FGFR) was assessed (Figure 4.2). An increased downstream response following activation of the mutant receptors compared to the wild type

receptor could be indicative of the increased receptor affinity for ligand, since it would lead to engagement of more receptors at any given ligand concentration and hence increased downstream signalling. It has previously been demonstrated that increased affinity of the mutant receptors for a particular FGF ligand correlates with an enhanced mitogenic activity of cells expressing these receptors upon exposure to this ligand [160], which means that changes in the magnitude of Erk1/2 activation may be indicative of changes in receptor affinity for ligand.

The wild type receptor only activated Erk1/2 in the presence of more than 5ng/ml FGF9, and maximal Erk1/2 activation was achieved in the presence of 10ng/ml FGF9 (Figure 4.2A). In contrast, the S252W mutant receptor led to almost the same level of Erk1/2 stimulation induced by the wild type FGFR2 in the presence of 10ng/ml when only 0.5ng/ml FGF9 were added. This finding correlates with previous reports of increased affinity of the S252W mutant FGFR2 for ligand, since significantly lower amounts of ligand were required to induce the same level of Erk1/2 phosphorylation. The enhanced Erk1/2 response to FGF9 stimulation was observed throughout the dose response curve, with an increase in FGF9 dose corresponding to an increase in Erk1/2 phosphorylation. Interestingly, the level of Erk1/2 activation in cells expressing the wild type FGFR2 never reached the level of Erk1/2 activation achieved by engagement of the mutant receptors, even when significantly more than 10ng/ml FGF9 were used (data not shown). This indicates that the two mutations must introduce other changes in terms of receptor functionality and signal transduction that cannot be explained by a simple increase in receptor affinity for FGF ligand.

The P253R mutant has also been reported to have a higher affinity for FGF ligand. However it was difficult to compare the dose response curve obtained after FGF9 stimulation with that obtained for the wild type FGFR2 because expression of the P253R receptor resulted in some Erk1/2 phosphorylation in unstimulated cells. In comparison, the other two receptors did not cause any such effect (Figure 4.2). It is unclear whether this is a result of the increased basal P253R receptor phosphorylation (refer to section 4.2.5), but it meant that no direct comparison in terms of activation by lower doses could be made in this case. When deducting the basal Erk1/2 phosphorylation level, the pattern observed in cells expressing the P253R receptor is similar to that of the wild type receptor.

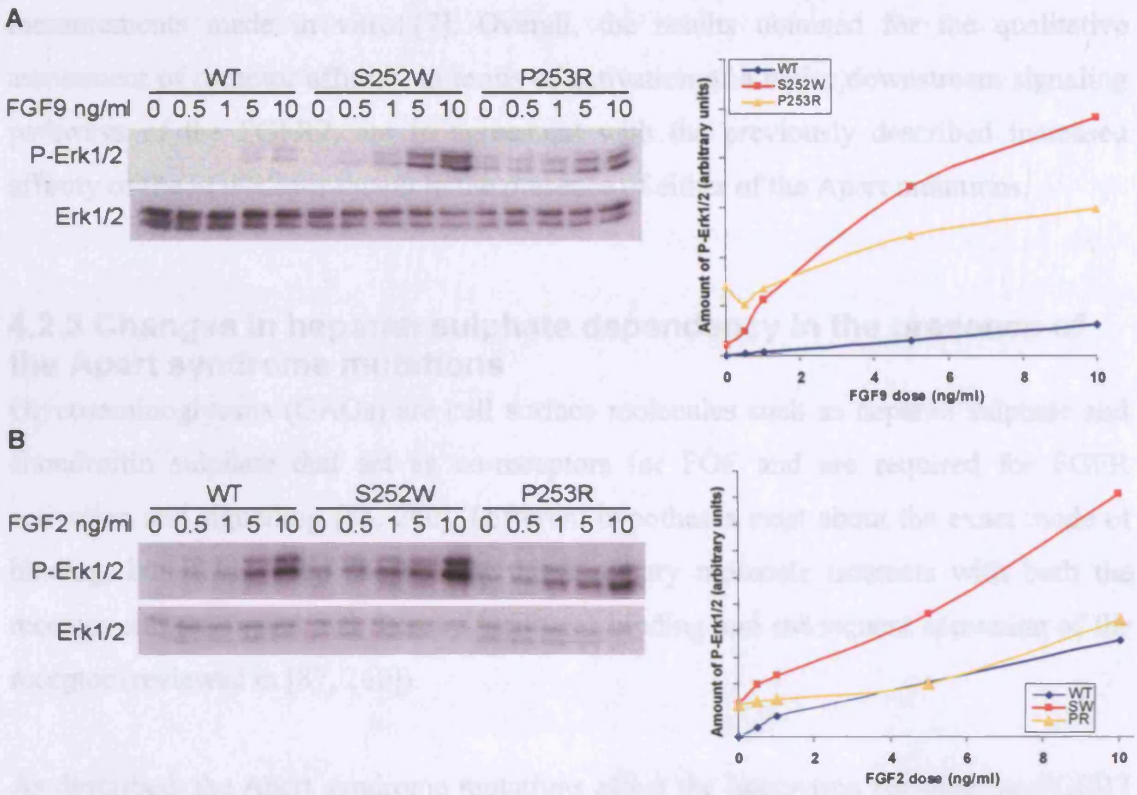


Figure 4.2: Effects of different FGF doses on Erk1/2 activation in cells expressing wild type or Apert mutant receptors HEK 293T cells were serum-starved overnight and stimulated with various concentrations of FGF9 (A) or FGF2 (B) for ten minutes. 200µg of cell lysate were subjected to SDS-PAGE and immunoblotting with an anti-phospho-Erk1/2 antibody. To assess equal loading, blots were stripped and re-probed with an anti-Erk1/2 antibody. The amounts of phospho-Erk1/2 were assessed by densitometry and represented graphically for each experiment. The results shown are representative of three independent experiments.

Because the activation of the wild type receptor and its ability to activate Erk1/2 are quite low, the same experiment was carried out using FGF2 instead of FGF9. A very similar trend was observed upon stimulation of cells with FGF2 as with FGF9 (Figure 4.2B). The higher level of phosphorylation in cells expressing the wildtype receptor is likely due to the activation of endogenous FGFRs as well as the over-expressed FGFR2 or may reflect the increased affinity of the FGFR2 for FGF2 compared to FGF9 [7]. Despite the higher level of Erk1/2 phosphorylation following wild type FGFR2 activation, both mutant receptors still portrayed an enhanced level of Erk1/2 phosphorylation at lower doses of FGF2. This is consistent with the increased affinity of the Apert mutant receptors for ligand. The effect was more pronounced for the S252W receptor compared to the P253R receptor, which seems to indicate that the S252W mutation results in a greater affinity increase in binding of the FGFR2 to both FGF2 and FGF9. This observation is also in agreement with previously published affinity

measurements made in vitro [7]. Overall, the results obtained for the qualitative assessment of receptor affinity, in terms of activation of a major downstream signaling pathways of the FGFR2, are in agreement with the previously described increased affinity of the FGFR2 for ligand in the presence of either of the Apert mutations.

4.2.3 Changes in heparan sulphate dependency in the presence of the Apert syndrome mutations

Glycosaminoglycans (GAGs) are cell surface molecules such as heparan sulphate and chondroitin sulphate that act as co-receptors for FGF and are required for FGFR activation and signalling [86, 260]. Different hypotheses exist about the exact mode of binding, but it has been shown that this auxiliary molecule interacts with both the receptor and the ligand and thereby facilitates binding and subsequent activation of the receptor (reviewed in [87, 260]).

As described, the Apert syndrome mutations affect the interaction between the FGFR2 and FGF ligand. Since a complex between FGF, GAGs and the FGFR is required for efficient activation of the receptor, it was of interest to investigate whether the Apert syndrome mutations also affect the interaction of the FGFR2 with GAGs. Changes in this interaction would have further effects on activation of the mutant FGFR2 and might contribute to the development of the Apert phenotype. It was proposed that the Apert mutations might not only affect affinity of the receptor for FGF but also its dependency on GAGs for activation. An investigation of the requirement for heparan sulphate (HS) for activation of the Erk1/2 pathway by the wild type and the two Apert mutant receptors was undertaken. The stimulation of Erk1/2 by FGF2 was not dependent on the presence of HS and the amount of phosphorylated Erk1/2 changed little over the range of doses of HS from 0μg/ml to 5μg/ml (Figure 4.3A). This is likely due to the presence of high levels of HSPG on the cell surface or in the cell medium [261], which may be sufficient to allow interaction of FGF2 with either endogenous FGFRs or the exogenously expressed WT-FGFR2-GFP. Since FGF2 is not specific for the FGFR2, it is likely that the high level of Erk1/2 activation observed is also, at least partially, due to binding of FGF2 to and subsequent activation of other FGFRs expressed in HEK 293T cells.

Interestingly the stimulation of WT-FGFR2-GFP by FGF9 was strongly dependent on HS being present. Without HS, Erk1/2 was minimally phosphorylated, and only in the presence of 0.5-1μg/ml HS was FGF9 able to invoke Erk1/2 activation. This stands in contrast to the activation by FGF2. This observation may be explained by the fact that the amount of HSPG normally present on the cell surface is not great enough to allow sufficient FGF9 to complex with the exogenously expressed WT FGFR2, or that FGF9 has a greater requirement for HS to allow efficient complex formation with the receptor. FGF9 has a slightly lower affinity for FGFR2 than FGF2 [7], which may also explain the greater requirement for HS to facilitate FGF9:FGFR2 complex formation.

Figure 4.3: The role of heparan sulphate in Erk1/2 activation HEK 293T cells expressing the wild type FGFR2 were serum-starved overnight and stimulated with 10ng/ml FGF2 or FGF9 for ten minutes in the presence of various amounts of heparan sulphate (HS) as indicated. 200μg of cell lysate were subjected to SDS-PAGE and immunoblotting with an anti-phospho-Erk1/2 antibody and an anti-Grb2 antibody to assess equal loading (A). HEK 293T cells expressing the wild type, S252W or P253R receptors were exposed to FGF9 (ten minutes), HS (five minutes) or HS followed by FGF9 (five minutes followed by ten minutes). The amount of phospho-Erk1/2 present was assessed by western blotting (B). An anti-Grb2 antibody was used to show equal loading.

To investigate whether HS was also required for Erk1/2 activation following stimulation of the Apert mutant receptors with FGF9, all three HEK 293T clonal cell lines were exposed to FGF9 in the absence or presence of additional heparan sulphate (Figure 4.3B). Interestingly, the activation of S252W and P253R FGFR2 by FGF9 was not dependent on the presence of additional HS (Figure 4.3B: S252W and P253R, lane 2

versus lane 4). The level of Erk1/2 phosphorylation achieved in the presence of HS (Figure 4.3B lane 4 S252W and P253R) was comparable to the levels achieved when only FGF9 was used to stimulate the cells (Figure 4.3B lane 2 S252W and P253R). This is in contrast to the wild type receptor, which required addition of HS before significant Erk1/2 phosphorylation could be detected. Thus the Apert mutations not only result in increased affinity for FGF, but also affect the dependency of the FGFR2 on HS for its activation.

4.2.4 Changes in receptor glycosylation as a result of Apert syndrome mutations

One effect of the Apert syndrome mutations on the FGFR2 itself became obvious during routine western blot analysis of the receptor. All three variants of the GFP-tagged FGFR2 appeared as three bands when whole cell lysates from HEK 293T cells were subjected to SDS-PAGE (Figure 4.1A). The uppermost bands of the S252W and P253R receptors portrayed slightly reduced electrophoretic mobility, whereas the other two bands appeared to be of the same relative molecular weight as the equivalent bands of the wild type receptor. Interestingly, the two bands of lower molecular weight were less apparent in the case of the Apert mutant receptors, particularly the FGFR2 containing the S252W mutation. A similar trend was also observed in PC12 cells, although the two lower bands were not always apparent (refer to Figure 3.7A). Various RTKs run as smears or several bands on SDS-PAGE gels, as a result of the presence of differentially glycosylated forms [262, 263]. In addition to FGFR selectivity for specific FGFs and glycosaminoglycans as well as the assembly of FGFR:ligand complexes with different signalling potential, N-glycosylation of the FGFR has been proposed as an additional regulatory factor of FGFR signaling [162]. Changes in receptor glycosylation in the presence of the Apert mutations may therefore be an important factor resulting in alterations in signal transduction from the mutant receptors. To investigate whether the altered electrophoretic mobility of the S252W and P253R receptors was due to changes in N-glycosylation, cells were grown in the presence of tunicamycin (an antibiotic that acts as a specific inhibitor of N-glycosylation). This revealed that indeed the lowest of the three bands corresponds to the unglycosylated form of the receptor (Figure 4.4A), which identifies the middle band as an intermediately glycosylated form (under-glycosylated) and the highest band as the fully glycosylated receptor. It also confirmed

that the differences observed between the wild type and mutant receptors were due to additional glycosylation.

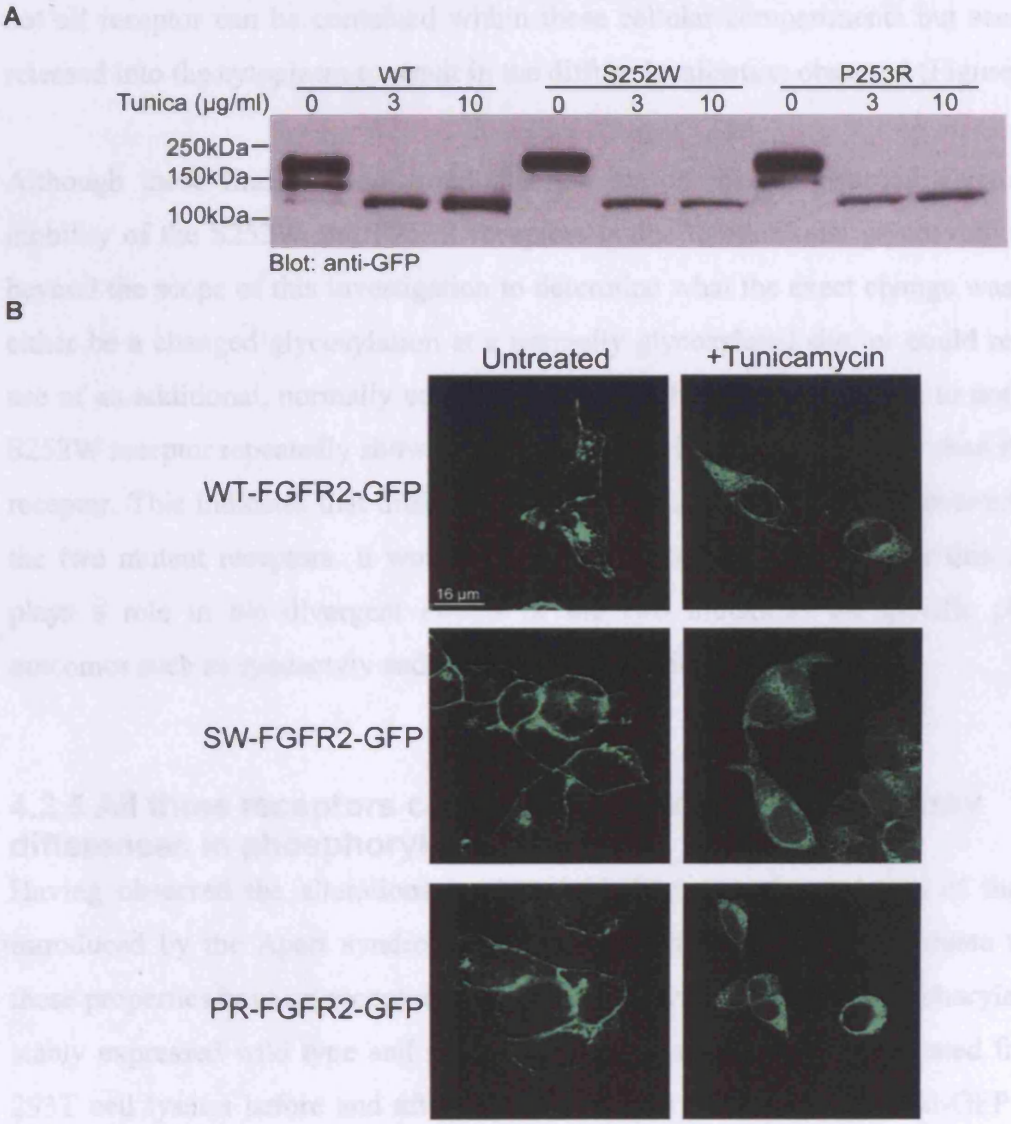


Figure 4.4: Altered receptor glycosylation in the presence of the Apert syndrome mutations HEK 293T cells were incubated with 3 or 10μg/ml tunicamycin overnight. 200μg of whole cell lysate were subjected to immunoblotting with an anti-GFP antibody to assess changes in electrophoretic mobility of the receptors (A). Cells grown in the presence of 10μg/ml tunicamycin overnight were also assessed by confocal microscopy for the changes in cellular distribution in the absence of the glycosylation after overnight treatment with 10μg/m tunicamycin and fixation in 4% paraformaldehyde (B).

Confocal microscopy of cells treated with tunicamycin revealed that the unglycosylated receptor did not localise to the plasma membrane and was instead located diffusely throughout the cytoplasm (Figure 4.4B). It has been shown previously that only the fully glycosylated form of the FGFR is localised at the plasma membrane [262, 263].

Accordingly, only the fully and perhaps the under-glycosylated forms of the receptor are targeted to the correct cellular localisation. The unglycosylated FGFR2 is most likely the form found in the ER/Golgi in growing cells. In the presence of tunicamycin, not all receptor can be contained within these cellular compartments but seems to be released into the cytoplasm to result in the diffuse localisation observed (Figure 4.4B).

Although these findings confirmed that the reason for the retarded electrophoretic mobility of the S252W and P253R receptors is due to additional glycosylation, it was beyond the scope of this investigation to determine what the exact change was. It could either be a changed glycosylation at a normally glycosylated site, or could result from use of an additional, normally unglycosylated site. It is also interesting to note that the S252W receptor repeatedly showed the greater shift in molecular weight than the P253R receptor. This indicates that differences in glycosylation may even be present between the two mutant receptors. It would be of interest to elucidate whether this difference plays a role in the divergent effects of the two mutations on specific phenotypic outcomes such as syndactyly and cleft palate formation [264].

4.2.5 All three receptors can be phosphorylated and portray differences in phosphorylation pattern

Having observed the alterations in ligand binding and glycosylation of the FGFR2 introduced by the Apert syndrome mutations it was chosen to investigate the effect these properties have on receptor dimerisation, activation and autophosphorylation. The stably expressed wild type and mutant receptors were immunoprecipitated from HEK 293T cell lysates before and after stimulation with FGF9 using an anti-GFP antibody (Figure 4.5). All three receptors were able to undergo dimerisation and *trans*-autophosphorylation, as indicated by the increased receptor phosphorylation upon exposure to FGF9 (Figure 4.5B). Since the GFP-tag was present on all receptors the differences observed were not due to addition of the tag but must represent intrinsic differences in the receptors due to the presence of the Apert syndrome mutations. The ability of all three receptors to undergo autophosphorylation upon addition of ligand indicates that the GFP-tag does not interfere with receptor functionality.

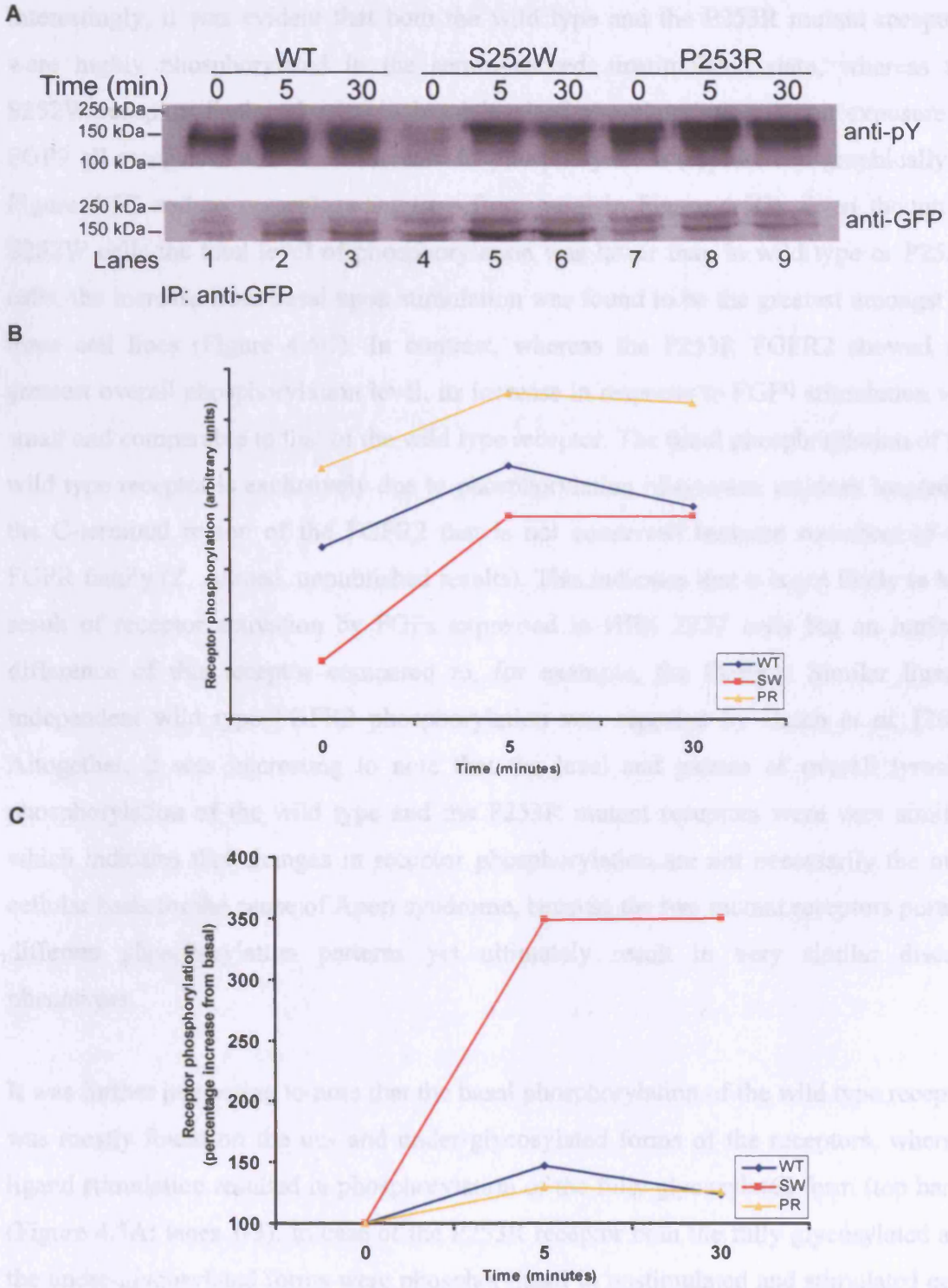


Figure 4.5: Phosphorylation of GFP-tagged wild type and mutant receptors (A) HEK 293T cells expressing the three respective receptors were serum starved overnight, stimulated with 10ng/ml FGF9 and lysed. 2mg of cell lysate were subjected to immunoprecipitation with an anti-GFP antibody. The western blot was probed with an anti-phosphotyrosine antibody, stripped and re-probed with an anti-GFP antibody. The levels of receptor phosphorylation were quantified using densitometry and represented graphically (B) or as a percentage increase from each respective 'Basal' level (C). The results are representative of two independent experiments.

Interestingly, it was evident that both the wild type and the P253R mutant receptors were highly phosphorylated in the serum-starved, unstimulated state, whereas the S252W receptor displayed a lower basal level of phosphorylation. Upon exposure to FGF9 all receptors showed an increase in phosphorylation (represented graphically in Figure 4.5B and as percentage increase from basal in Figure 4.5C). Even though in S252W cells the total level of phosphorylation was lower than in wild type or P253R cells, the increase from basal upon stimulation was found to be the greatest amongst all three cell lines (Figure 4.5C). In contrast, whereas the P253R FGFR2 showed the greatest overall phosphorylation level, its increase in response to FGF9 stimulation was small and comparable to that of the wild type receptor. The basal phosphorylation of the wild type receptor is exclusively due to phosphorylation of tyrosine residues located in the C-terminal region of the FGFR2 that is not conserved between members of the FGFR family (Z. Ahmed, unpublished results). This indicates that it is not likely to be a result of receptor activation by FGFs expressed in HEK 293T cells but an intrinsic difference of this receptor compared to, for example, the FGFR1. Similar ligand-independent wild type FGFR2 phosphorylation was reported by Hatch *et al.* [263]. Altogether, it was interesting to note that the level and pattern of overall tyrosine phosphorylation of the wild type and the P253R mutant receptors were very similar, which indicates that changes in receptor phosphorylation are not necessarily the only cellular basis for the cause of Apert syndrome, because the two mutant receptors portray different phosphorylation patterns yet ultimately result in very similar disease phenotypes.

It was further interesting to note that the basal phosphorylation of the wild type receptor was mostly found on the un- and under-glycosylated forms of the receptors, whereas ligand stimulation resulted in phosphorylation of the fully glycosylated form (top band) (Figure 4.5A: lanes 1-3). In case of the P253R receptor both the fully glycosylated and the under-glycosylated forms were phosphorylated in unstimulated and stimulated cells (Figure 4.5: lanes 4-6). In contrast, ligand stimulation of the S252W receptor resulted mostly in phosphorylation of the fully glycosylated form (Figure 4.5: lanes 7-9). The unglycosylated form of the receptor is not found at the plasma membrane (Figure 4.4B), and its phosphorylation may be due to increased dimerisation as a result of the lack of glycosylation [263]. Primarily the fully glycosylated FGFR1 was found to be targeted to the plasma membrane in BaF3 cells [262], but it is not clear whether the intermediately glycosylated FGFR2 is located at the plasma membrane in HEK 293T cells or not.

Since phosphorylation of this form of the FGFR2 did not significantly change following FGF9 stimulation and is therefore likely to only consist of phosphorylation of the C-terminal residues described, it may not contribute largely to signal transduction from the FGFR2. Altogether, the data presented indicate that the changes in receptor glycosylation may affect the receptor phosphorylation patterns. Such changes would then be expected to result in altered intracellular signalling downstream of the mutant receptors compared to the wild type FGFR2.

4.2.6 The unglycosylated FGFR2 can still be phosphorylated but does not activate Erk1/2

After having observed the differences in phosphorylation of the differently glycosylated forms of the receptor, the question arose of whether this effect was due to the mutations alone or whether they were the result of the additional glycosylation of the S252W and P253R receptors. To address this question, cells expressing each of the receptors were incubated with tunicamycin and the phosphorylation pattern of receptors was assessed by immunoblotting with an anti-phosphotyrosine antibody. The unglycosylated receptors could still be phosphorylated, but differences between the three receptor types were observed. Hatch *et al.* recently reported that the unglycosylated FGFR2 was more strongly phosphorylated in the absence of ligand than the fully glycosylated receptor [263]. This effect was mirrored by the wild type FGFR2-GFP expressed in HEK 293T cells in this study (Figure 4.6), which may explain the high level of basal phosphorylation observed (Figure 4.5A). In contrast, both of the unglycosylated mutant receptors were not phosphorylated as strongly as the unglycosylated wild type receptor. The P253R mutant portrayed a similar level of phosphorylation in its fully glycosylated and the unglycosylated state. The S252W mutant receptor seemed to be less phosphorylated in tunicamycin-treated than in untreated cells. This is in agreement with the low basal phosphorylation of this receptor and the fact that the S252W receptor was only found to be phosphorylated in its fully glycosylated state as described previously (Figure 4.5A). The under-glycosylated receptor was strongly phosphorylated to comparable levels in all three cell lines and was still detected in tunicamycin treated cells by the anti-phosphotyrosine antibody (middle band as indicated). However, it could not be detected using the anti-GFP-antibody. This indicates that only a small amount of the under-glycosylated receptor is present, which is, however, highly phosphorylated. The level of phosphorylation of this form of the receptor decreased

upon tunicamycin treatment in accordance with the decrease in its amount present as a result of the inhibition of N-glycosylation.

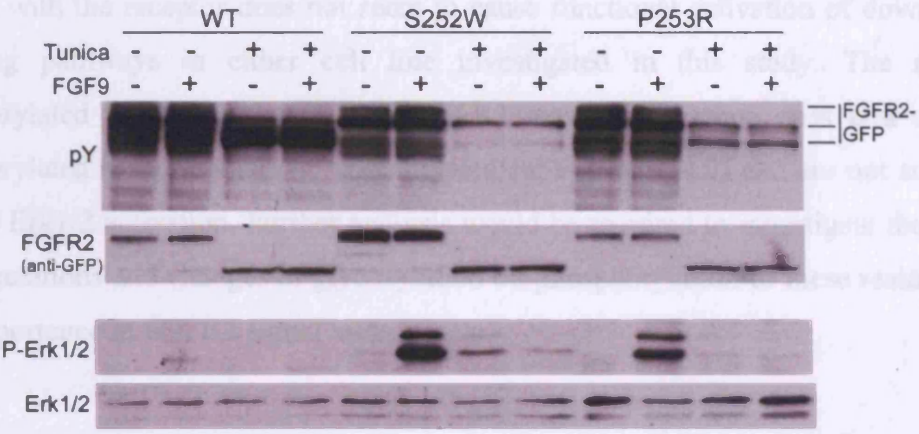


Figure 4.6: Lack of FGFR2 glycosylation inhibits Erk1/2 activation but not receptor phosphorylation HEK 293T expressing the wild type, S252W or P253R FGFR2 were treated with 10μg/ml tunicamycin for 24 hours or left untreated, serum-starved overnight and stimulated with 10ng/ml FGF9. Cells were lysed and 200μg cell lysate were subjected to SDS-PAGE and immunoblotting with an anti-phosphotyrosine and anti-phospho-Erk1/2 antibody. The western blot was stripped and re-probed with anti-GFP and anti-Erk1/2 antibodies to assess receptor expression levels and equal loading respectively.

An increase in FGFR2 dimerisation and tyrosine phosphorylation in the absence of N-glycosylation has been described previously [263]. It seems that the Apert mutations not only affect the receptor glycosylation, but also affect this ligand-independent dimerisation in the absence of N-glycosylation described by Hatch *et al.* [263]. Neither the S252W nor the P253R receptors were as strongly phosphorylated in the unglycosylated state as the wild type receptor (Figure 4.6). However, this effect seems to be reversed in the presence of the additional glycosylation in the fully glycosylated receptor, since this form was more strongly phosphorylated in the Apert mutant receptors compared to the wild type FGFR2 (Figure 4.5).

The unglycosylated wild type FGFR2 has been reported to portray increased association with PLCγ and FRS2 [263]. In light of this finding as well as the observation that the unglycosylated receptors were differentially phosphorylated, their ability to activate Erk1/2 in the absence and presence of FGF9 was analysed. Whereas the untreated receptors portrayed a stimulation-dependent increase in Erk1/2 phosphorylation, this was absent in the tunicamycin-treated cells, which is in agreement with the lack of

membrane localisation of the unglycosylated receptors (Figure 4.4). This lack of Erk1/2 phosphorylation is interesting in light of the increased association of PLCγ and FRS2 with the unglycosylated receptor described previously [263]. The association of these proteins with the receptor does not seem to cause functional activation of downstream signalling pathways in either cell line investigated in this study. The residues phosphorylated on the unglycosylated FGFR2 may be the same ones that are also phosphorylated in serum-starved, unstimulated cells (Figure 4.2) and are not sufficient to cause Erk1/2 activation. Further analysis would be required to investigate the effects of the mutations and changes in glycosylation on phosphorylation of these residues and their importance in FGFR2 signal transduction.

4.3 Discussion

The work presented in this chapter demonstrates the vast number of effects that the Apert syndrome mutations have on various properties of the FGFR2 such as activation by FGF and heparan sulphate, receptor phosphorylation and glycosylation. All of these properties are important in regulating receptor activation and function, and their disruption as a result of a single point mutation (S252W or P253R) may form the basis of the manifestation of Apert syndrome.

4.3.1 Effects of the Apert syndrome mutations on activation of the FGFR2 by FGF ligand

It has been reported previously that the Apert syndrome mutations in FGFR2 result in increased affinity for FGF ligand [7, 161]. The data presented in Figure 4.2 indicate that the effect observed in vitro is partially mirrored in vivo. The S252W mutation causes a greater affinity increase than the P253R receptor in vitro, an effect that was mirrored by an increase in the level of activation of Erk1/2 in vivo. The S252W receptor was able to induce stronger Erk1/2 phosphorylation at lower doses of both FGF9 and FGF2. This seems to be in agreement with the relative affinity for these ligands, since the higher the affinity the more receptor:ligand complexes can be formed even at relatively low ligand concentrations. The increased number of active receptors would then result in increased activation of the Erk1/2 pathway downstream of the FGFR2. However, the addition of excess amounts of FGF9 to cells expressing the wild type FGFR2 was not able to result in the same level of Erk1/2 phosphorylation that could be reached upon stimulation of the S252W or P253R receptors. This indicates that the increased activation of Erk1/2 downstream of the mutant receptors is not simply due to an increased affinity of the receptors for ligand, but that additional changes in the receptor and signalling from it also contribute to the greatly enhanced activation of this downstream kinase observed.

The main study of the effects of the Apert mutations on FGFR2 affinity for various FGF ligands has been undertaken using only part of the extracellular domain expressed in bacteria. The lack of the first Ig-like domain (D1), glycosylation and the absence of GAGs means that this system lacks many of the factors that are important in regulating ligand binding. The comparison of the interaction of the wild type and mutant FGFR2 with ligand using only parts of the extracellular domain expressed in bacteria means that both glycosylation and the requirement for GAGs were not taken into account [7, 163].

Nonetheless, the results presented in this chapter indicate that the general trend of increased affinity for FGF ligand seems to be mirrored by the activation of Erk1/2 at lower doses and generally enhanced Erk1/2 phosphorylation in cells expressing the Apert mutant receptors compared to the wild type FGFR2. This is an interesting trend in light of recent reports indicating that lower affinity ligands for the EGFR such as E4T and TGFα result in greater mitogenic signals compared to the high affinity ligand EGF. This dimished EGFR-induced signalling upon stimulation with a high affinity ligand was found to be due to relatively greater levels of receptor ubiquitination, internalisation and degradation [265-267]. These observations further indicate that in the case of the Apert syndrome mutations, which result in increased affinity of the receptor for FGF ligand and enhanced activation of downstream signalling, other factors must contribute to this phenomenon.

4.3.2 The Apert syndrome mutations alter the heparan sulphate dependency of the FGFR2

In addition to the increased affinity of the Apert mutant receptors for FGF ligand, it was interesting to note that the wild type receptor portrayed HS dependence for activation by FGF9 in HEK 293T cells, although this cell type is likely to express various GAGs [261]. The two mutant receptors did not require the presence of additional HS to cause activation of Erk1/2 and the addition of HS did not enhance its phosphorylation. This was in agreement with a previous report indicating that the S252W receptor is less selective for different GAGs (i.e. does not require a specific type of GAG for receptor activation) and requires lower doses of GAGs for activation [261]. The data presented in Figure 4.5 indicate that the activation of the mutant receptors by FGF9 is HS independent. Although McDowell *et al.* [261] reported a slight increase in mitogenesis upon the addition of various GAGs to the S252W receptor and FGF9, this increase (especially by HS) was minimal and the mitogenic response in the absence of any GAG was already almost as great as the highest level reached by the wild type receptor in the presence of large amounts of HS. This observation supports the HS-independent activation of the mutant receptors observed in HEK 293T cells in this study. Some enhancement of receptor activation by FGF9 may take place by endogenous molecules, but the dependence on additional HS that was observed for the wild type FGFR2 was not mirrored by either the S252W or the P253R receptor. It is also interesting to note, that stimulation of the wild type receptor with FGF2 was not affected by the addition of

external HS. FGF2 has a higher affinity for both the FGFR2 and other FGFRs that might be expressed in HEK 293T cells [5] and has been shown to require less GAGs and show less specificity for individual GAGs for activation of mitogenesis [261], which may explain the results observed.

The data presented support the previously described enhanced affinity of the Apert mutant receptors for ligands such as FGF9. In addition, both the S252W and the P253R mutations were observed to affect the requirement for HS for receptor activation. It is possible that this effect is a result of full receptor glycosylation in contrast to bacterially expressed extracellular domains. It seems that the mutations alter the selectivity for specific GAGs, and therefore those molecules normally present on HEK 293T cells are sufficient to allow maximum activation of Erk1/2 by FGF9 in the absence of additional HS. Previous studies have indicated that the protein composition of the extracellular matrix surrounding cells with the Apert syndrome mutations is altered [268]. Such changes could further enhance the increased activation of the mutant receptors compared to the wild type receptor and form an additional factor that contributes towards development of Apert syndrome in the whole organism. Expression of different proteoglycans on, or surrounding, specific cells may enhance and/or affect the change in affinity for FGF observed in Apert mutant receptors and may therefore affect the signal created intracellularly. Especially if the mutant receptors do not rely on the presence of a specific GAG for activation, this could lead to a greater signal enhancement effect. The lesser requirement/selectivity for certain GAGs would allow activation of the mutant receptors in a cellular context in which perhaps activation was normally prevented because the specific GAG for FGF:FGFR complex formation is not expressed on the surface of these cells. This would mean that in the context of the whole organism a major component controlling when and where a certain receptor is activated is lost and incorrect signalling may ensue as a result.

It would be interesting to investigate the GAG dependence of the wild type and mutant receptors in the presence of various other FGFs to determine whether this adds further complexity to the way in which Apert syndrome develops on the organismal level. Additionally, experiments such as isolating the glycosylated extracellular domains of the FGFR2 and the S252W and P253R mutants as described by Duchesne *et al.* for the FGFR1 [162] and measuring their affinity for heparin, HS or other GAGs are required to determine whether the altered glycosylation of the mutant receptors affects the

dependency on various GAGs of these receptors or whether this property is a result of the amino acid substitutions and subsequent conformational changes alone. The amino acid substitutions have been shown not to affect the structure of the FGFR2 extracellular domain greatly [163]. However, one could propose that the additional glycosylation of the S252W and P253R receptors results in a more favourable conformation of this region and thereby alleviates the requirement for GAGs for FGF ligand binding.

4.3.3 The Apert mutations result in altered, not simply enhanced, receptor autophosphorylation patterns

A logical proposition based on the increased affinity for FGF and the observed changes in Erk1/2 activation by the Apert mutant receptors would be that the phosphorylation levels of these receptors were simply enhanced. Contrary to this hypothesis, the overall phosphorylation pattern of the mutant receptors, in particular that of the S252W receptor, was significantly different from the wild type FGFR2 and did not reflect a simple increase concomitant with the changes in receptor/ligand interaction. Thus the Apert mutations introduce changes in the FGFR2 other than just enhanced affinity for FGFs. If only the affinity for ligand was altered, then one would expect that more of the higher affinity receptors were occupied by ligand within a given period of time than of the lower affinity (i.e. wild type) receptor. This would ultimately result in a greater number of receptors being phosphorylated, which would be represented by an overall increase in receptor phosphorylation in an immunoprecipitation experiment. However, this was not the case, and the Apert mutations altered the overall phosphorylation pattern in both the ligand-independent and ligand-stimulated states. Particularly the lower basal phosphorylation of the S252W receptor indicates that the changes in the extracellular region, namely the point mutation and the altered glycosylation, are somehow transmitted to the intracellular region where they affect phosphorylation of tyrosine residues possibly by changing the accessibility of certain residues by the kinase domain. The high basal phosphorylation of the wild type receptor is the result of phosphorylation of two tyrosine residues at the distal C-terminus that are not conserved amongst the other members of the FGFR family (Z. Ahmed, unpublished results). This indicates that some structural changes must occur in the intracellular region to prevent phosphorylation of these residues when the S252W mutation is present. Similarly, the increased basal phosphorylation observed in the case of the P253R receptor must be the

result of structural changes that allow greater phosphorylation of these and possibly additional residues. A large amount of the basal phosphorylation of the wild type receptor was due to phosphorylation of the unglycosylated and under-glycosylated receptor. In contrast, this was completely absent in the S252W receptor. In case of the P253R receptor, both fully glycosylated and under-glycosylated receptors were highly phosphorylated in the unstimulated state. It would be interesting to elucidate the exact difference in glycosylation between the wild type and the S252W receptor and whether once this additional/altered glycosylation (rather than total glycosylation in the presence of tunicamycin) is abolished, the phosphorylation pattern of the mutant receptors resembles that of the wild type receptor or whether the mutations themselves are responsible for the observed differences. Although the altered glycosylation seems to play an important role in the changes in receptor phosphorylation, the unglycosylated mutant receptors were both phosphorylated to a lesser extent than the wild type receptor. This indicates that the mutations themselves also play a role in the observed changes in receptor tyrosine phosphorylation.

It was beyond the scope of this work to analyse whether specific tyrosine residues are differentially phosphorylated in the wild type and the mutant receptors after FGF stimulation, and further experiments are required to find out whether this is the case. Only the tyrosines in the activation loop (Y653 and Y654) and Y766 have truly been implicated with a function in FGFR1 signalling, and somewhat minor functions for other residues have been proposed [15, 103, 122]. It would be extremely interesting to elucidate any specific differences in the stoichiometry of phosphorylation of specific tyrosine residues as a result of the Apert syndrome mutations and whether residues that remain unphosphorylated in the wild type receptor are phosphorylated in receptors containing either of the two Apert syndrome mutations. Such differences might result in different recruitment of signalling proteins to the receptor. Point mutations of individual tyrosine residues or several residues in combination might reveal the basis for such altered protein recruitment that may lead to enhanced Erk1/2 activation. The FGFR2 contains several tyrosine residues in its C-terminal tail that are not conserved between the different members of the FGFR family, which may not only form the basis of the high level of basal phosphorylation observed but may also have functional roles in the differential activation of downstream signalling pathways in cells expressing the wild type and mutant receptors.

4.3.4 Changes in receptor phosphorylation may affect other receptor properties such as its downregulation by endocytosis

Clear differences in the basal and ligand-stimulated receptor phosphorylation of all three receptors were observed, which did not correlate with the relative affinities of the different receptors for FGF ligand. It therefore seems that mechanisms other than simple enhancement of the 'regular' signalling pathways play an important role in causing enhanced Erk1/2 activation and possibly affecting other signalling pathways. The P253R mutation is of particular interest since it caused increased basal receptor phosphorylation, but the percentage increase in phosphorylation from basal upon FGF9 stimulation was of a similar magnitude as that of the wild type receptor (Figure 4.5). This means that the overall increased phosphorylation is only due to the changes that are already present in the receptor in the absence of ligand (i.e. higher basal receptor phosphorylation). In addition, the activation of Erk1/2 with varying doses of FGF portrayed a similar pattern (Figure 4.1). This indicates that increased affinity and hence increased receptor phosphorylation are not the only effects of the Apert syndrome mutations on the P253R receptor. Similarly, the S252W receptor portrayed greatly decreased basal tyrosine phosphorylation, which indicates that other factors must affect this receptor and subsequently its signalling ability. The relatively lower phosphorylation of this receptor following FGF9 stimulation also shows that increased receptor affinity for FGF ligand is not mirrored by increased phosphorylation and that other mechanisms must contribute to the enhanced activation of pathways such as the Erk1/2 pathway at lower doses of FGF ligand.

Indeed, work in our laboratory has shown that the localisation pattern of both the S252W and the P253R was altered in response to FGF9 stimulation in HEK 293T cells ([224], in submission). Both mutant receptors portrayed reduced levels of endocytosis, which would be expected to play an important role in downstream signalling, since enhanced presence at the membrane or altered internalization of receptor/adaptor protein complexes would change the signal initiated from a receptor. This would explain how the activation of the Erk1/2 pathway could mirror increased affinity of the receptor for ligand despite receptor phosphorylation not following the same trend. It has been proposed that higher affinity ligands for the EGFR result in increased endocytosis and downregulation of this receptor [265, 267]. It is interesting to note that in the case of the Apert mutations this trend was not observed, and that indeed the opposite took place. Despite the increased affinity for FGF ligand, the S252W and P253R receptors

were retained at the membrane to a greater extent than the wild type FGFR2. This is likely to have an effect on the activation of downstream signalling pathways such as the Erk1/2 pathway. The reduced endocytosis may therefore play a role in resulting in the Erk1/2 activation pattern that mirrored the relative affinities of the three receptors for FGF9 (Figure 4.2).

It is unclear what mechanism mediates the altered internalisation pattern of the Apert mutant receptors, but the changes in phosphorylation pattern may play a significant role. Recent studies have implicated a kinase-regulated targeting signal in the cytoplasmic region of FGFR4 as an important mediator of whether the receptor is recycled or transported to the lysosome for degradation [269]. Additionally, activating mutations in the FGFR2 and FGFR3 have been shown (although somewhat conflictingly) to alter ubiquitination and receptor trafficking in various cell systems [171, 263, 270, 271]. These reports indicate that receptor trafficking could be affected by the altered phosphorylation patterns of the Apert mutant FGFR2 as well as changes in recruitment of proteins such as c-Cbl that are involved in receptor internalisation [114]. The removal of Y766 in the FGFR1, which is the binding site for PLCγ, inhibits receptor internalisation [272]. Hence it would be interesting to determine whether either phosphorylation of this residue is reduced/absent in the P253R receptor or whether PLCγ and/or c-Cbl recruitment to the P253R and/or S252W receptors is altered and thereby cause the changes in receptor localisation observed. However, alterations in receptor internalisation may not necessarily be the only determinant of Apert syndrome, since all three receptors did not portray high levels of internalisation in PC12 cells (data not shown). In PC12 cells, prolonged FGF stimulation results in differentiation into a neuronal type. To maintain responsiveness to FGF, downregulation of the FGFR would not be desired, which may explain why endocytosis of none of the receptors (including the wild type FGFR2) is observed in this cell line. Despite such cell-specific differences, it is clear that the Apert syndrome mutations affect various cellular properties in addition to the changes in ligand binding described previously and in this study. In combination, all of these effects may be important in causing changes in intracellular signalling from the FGFR2 that ultimately result in the manifestation of Apert syndrome.

4.3.5 The altered glycosylation pattern of the Apert mutant receptors may be an important regulator of the observed effects on the FGFR2

Although the mechanism by which the Apert syndrome mutations result in the changes in receptor phosphorylation, localisation and interaction with its two ligands is not entirely understood, it seems likely that the altered glycosylation of the receptor plays an important role in regulation of these properties. Glycosylation of proteins is often found at sites of change in the structure of proteins [273]. Thus it is possible that the substitution of residues 252 or 253 results in minor structural changes in the extracellular domain that lead to additional or different receptor glycosylation. This may then enhance and support a structural change, which would not have been observed in the crystal structure that was determined in the absence of glycosylation and that subsequently may affect various receptor properties. N-glycosylation sites on FGFRs are positioned such that they may affect interaction of the receptor with FGF or GAGs and receptor dimer and/or multimer formation [162]. The crystal structures of the extracellular domains of all three receptors was solved in the absence of glycosylation, which may explain why no significant structural differences could be observed [163]. Although reports have been published that claim that glycosylation of the FGFR does not affect its ligand binding abilities [274, 275], changes in glycosylation may have previously undescribed effects on the FGFR itself and signal transduction from it. This is particularly interesting in the light of the observed changes in receptor glycosylation of the Apert mutant receptors.

It has been shown previously that glycosylation is important for membrane localisation of tyrosine kinase receptors [101, 263], and tunicamycin treatment of cells inhibited localisation of the receptor to the cell membrane (Figure 4.4). All three FGFR2 constructs behaved the same way and were no longer found localised at the cell membrane in the absence of glycosylation. More importantly, correct glycosylation of the EGFR has been shown to be required for its normal endocytic behaviour [276]. This indicates that changes in the glycosylation of a receptor may affect the way in which it is internalised. The changes in FGFR2 glycosylation in the presence of the S252W or the P253R mutations might therefore be causing the differences observed in terms of receptor localisation compared to the wild type receptor. Further experiments are required to identify the exact differences in receptor glycosylation of the Apert mutant receptors, to elucidate how these effects might be brought about and whether removal of this additional glycosylation would restore normal receptor trafficking.

Studies elucidating the changes in receptor glycosylation that occur in the presence of the Apert syndrome mutations are also important to understand the way in which differences in receptor phosphorylation are initiated and whether they could be the result of structural changes imposed by altered receptor glycosylation. No clear evidence exists for the way in which altered glycosylation of the extracellular domain can affect the intracellular structure of a receptor. However, it has been proposed that a similar phenomenon takes place in the FGFR3 with an N328I mutation, which causes hypochondroplasia and is found within a putative glycosylation site in the extracellular domain [277]. Duchesne *et al.* superimposed some of the N-glycans structures identified by mass spectroscopy onto putative glycosylation sites in the two different crystal structures of the FGFR1/FGF/heparin complexes [162]. Some chains seemed to make extensive contact with receptor and/or ligand. Thus despite reports claiming that glycosylation does not alter/affect FGF binding [274, 275], the changes in glycosylation may affect the positioning of FGF, GAGs and FGFR in the complex. This could subsequently alter positioning of individual receptors in the dimer and may thereby transmit the differences between wild type and mutant receptors to the intracellular domain. This might then result in the differences in ligand-stimulated FGFR2 phosphorylation observed. Additionally, the lack of glycosylation was found to increase receptor phosphorylation and dimerisation of the wild type FGFR2 (Figure 4.6 and [263]). This indicates that glycosylation is an important regulator of these receptor properties and alterations in glycosylation may therefore also affect ligand independent receptor dimerisation and phosphorylation.

The possibility that the altered glycosylation of the mutant receptors might be an important regulator of receptor phosphorylation and localisation raises the important question of whether these alterations result in more changes in signalling from the receptor than a simple upregulation of receptor signalling on the basis of increased affinity for FGF. The results presented in Chapter 5 will focus on the changes in downstream signal transduction from and protein recruitment to the FGFR2 in the presence of the Apert syndrome mutations. Based on the data presented in this chapter, any effects observed may be due to altered glycosylation, phosphorylation, localisation or interplay between all of these properties.

4.3.6 Complex changes in the FGFR2 form the basis of Apert syndrome

Overall, the findings presented in this chapter and other work carried out in this laboratory ([224], in submission) demonstrate how mutations in the extracellular domain of receptors can affect their overall behaviour as well as its overall signalling capacity in various ways. Not only do the Apert mutations affect the ligand binding affinity of the receptor and the requirement for various GAGs for receptor activation, but they also affect receptor glycosylation. It is likely that all of these properties interact to affect receptor phosphorylation, localisation and activation of downstream targets. The precise contribution of the alteration in receptor glycosylation to the described effects in unclear and further analysis of the precise changes in glycosylation is required to elucidate its exact role. Nonetheless, the fact that in vivo the Apert mutations result in changes in addition to causing increased receptor affinity for ligand is an interesting observation that demands further investigation of the intracellular changes that occur consequently. Certainly these findings indicate that the mechanisms by which Apert syndrome is manifested in the whole organism are somewhat more complex than the previously reported increase in ligand binding and altered ligand recognition specificity of the FGFR2. The differences between wild type and mutant receptors described in this work indicate a further layer of complexity to the cellular basis of Apert syndrome and point out important factors whose integrity is required to initiate the correct cellular responses to FGFR2 activation.

Chapter 5

The Apert mutations affect signalling from the FGFR2 by altering protein recruitment

5.1 Introduction

The formation of multiprotein complexes upon activation of a given tyrosine kinase receptor has been implicated as an important factor in creating specificity in protein-protein interactions and ultimately in activation of downstream signalling pathways [126]. The precisely controlled assembly of proteins into complexes provides a mechanism for activation of numerous, specific downstream responses by regulating recruitment of specific individual proteins to activated receptors.

Mutations in any component of such multiprotein complexes would disrupt correct assembly and therefore ultimately affect the downstream signal. Nevertheless, the occurrence of mutations does not necessarily mean complete loss of all signal generated, depending on which component of the complex is mutated. The formation of complexes in addition to the existence of a degree of promiscuity in protein-protein interactions may allow for a signal to be generated to a certain extent even in the presence of mutations [126]. Nonetheless, mutations that affect regular protein-protein interactions, alter the enzymatic activity of kinases (without which phosphorylation and the creation of platforms for complex assembly is not possible) or that remove phosphorylatable tyrosines (thereby preventing protein recruitment via SH2 or PTB domains) may destroy normal formation of signalling complexes. The downstream signals initiated would be affected by mutation as a result of the altered protein recruitment to the receptor and disruption of protein complexes. Activation of downstream pathways might be completely lost or only the signal duration or strength may be affected, depending on the severity of complex disruption.

The Apert syndrome mutations are located in the extracellular domain of the receptor and therefore do not fall into the same category as any of the types of mutations mentioned above, i.e. they are not in a position where they would be expected to result in an altered binding site for adaptor proteins or directly interfere with the kinase activity of the receptor. Previous studies have described an affinity increase of the FGFR2 for FGF ligand in the presence of the Apert syndrome mutations [7, 161]. This would be expected to result in increased overall receptor phosphorylation because the increase in affinity for ligand means that more receptors can be activated. It might therefore be expected to observe only an increase but not a change in recruitment of adaptor proteins. Contrary to this hypothesis, the data presented in the previous chapter

described various changes incurred on the FGFR2 by the Apert syndrome mutations in addition to increased affinity for FGF ligand. The mutations resulted in altered interaction of the FGFR2 with both FGF and HS, and affected both the overall receptor phosphorylation as well as receptor glycosylation.

The observations described in the previous chapter indicated that the Apert syndrome mutations may affect intracellular signalling and protein recruitment despite being located in the receptor extracellular region. The changes observed in the Apert syndrome mutant receptors did not simply reflect an increased receptor phosphorylation due to the engagement and hence activation of more receptors because of increased affinity for FGF. It therefore seemed likely that protein recruitment to the receptor would also be altered instead of just being increased downstream of the S252W and P253R receptors. Subsequently, it was chosen to investigate the effect of these mutations on the activation of one of the main pathways downstream of the FGFR2, namely the Erk1/2 pathway, and on the recruitment of adaptor proteins. The results presented in this chapter focus on the adaptor protein Shc and the effects of the Apert mutations on its phosphorylation and cellular localisation patterns. The data presented, in conjunction with other findings made in the laboratory, describe the altered recruitment of an array of adaptor proteins to the FGFR2 as a result of the S252W and P253R mutations.

5.2 Results

5.2.1 Erk1/2 activation is enhanced by the S252W or P253R mutant FGFR2

The Erk1/2 pathway is one of the main signalling pathways activated downstream of the FGFR. It was therefore chosen to assess the effects of the Apert syndrome mutations on activation of this kinase to establish whether the altered tyrosine phosphorylation of each receptor was directly translated into downstream signalling pathways. All three cell lines were stimulated with FGF9 for various periods of time and whole cell lysates were subjected to immunoblotting with an anti-phospho-Erk1/2 antibody as a measure of its activation. HEK 293T cells do not respond to stimulation with FGF9, a type of FGF that portrays a fairly high level of specificity for FGFR2IIIc (although it can also activate FGFR3IIIc and FGFR4 [5]). Therefore background stimulation of endogenous pathways could be excluded (Figure 5.1C). In addition, although this cell line was reported to express FGFR2 mRNA [225], work in our laboratory failed to detect the protein by means of western blotting using an antibody specific for the FGFR2.

The dose response experiment in the previous chapter indicated that the level of Erk1/2 phosphorylation reached upon stimulation of either of the mutant receptors with FGF9 was much higher than that following activation of the wildtype receptor (Figure 4.2). To assess whether the Apert syndrome mutations also affected Erk1/2 activation over a prolonged period of stimulation, cells were stimulated with FGF9 for various time periods. The enhanced Erk1/2 phosphorylation persisted throughout the time course. Interestingly, the S252W receptor led to greater Erk1/2 activation in HEK 293T cells (Figure 5.1A), whereas in PC12 cells, expression of the P253R receptor portrayed the highest overall level of Erk1/2 activation (Figure 3.7B). These cell-specific differences may be due to each cell type expressing slightly different (levels of) signalling proteins. For example PC12 cells seem to express higher levels of the docking protein FRS2 than HEK 293T cells as assessed by western blotting. Differences in recruitment of such adaptor proteins by the two mutant receptors might be responsible for the different patterns observed in the two cell types.

In addition to generally increased levels of Erk1/2 activation, the onset of Erk1/2 phosphorylation was enhanced by the presence of the Apert mutations. In HEK 293T cells this was particularly the case for the S252W FGFR2, which reached near

maximum levels of Erk1/2 phosphorylation in response to FGF9 stimulation within five minutes of stimulation, whereas for the wild type receptor this only occurred after ten minutes of stimulation. Similar patterns were observed in HEK 293T cells stimulated with FGF2 (data not shown) and PC12 cells stimulated with FGF9 (Figure 3.7B). The faster onset of Erk1/2 activation in cells expressing the Apert mutant receptors is likely a result of the increased affinity of these receptors for FGF ligand [7, 161].

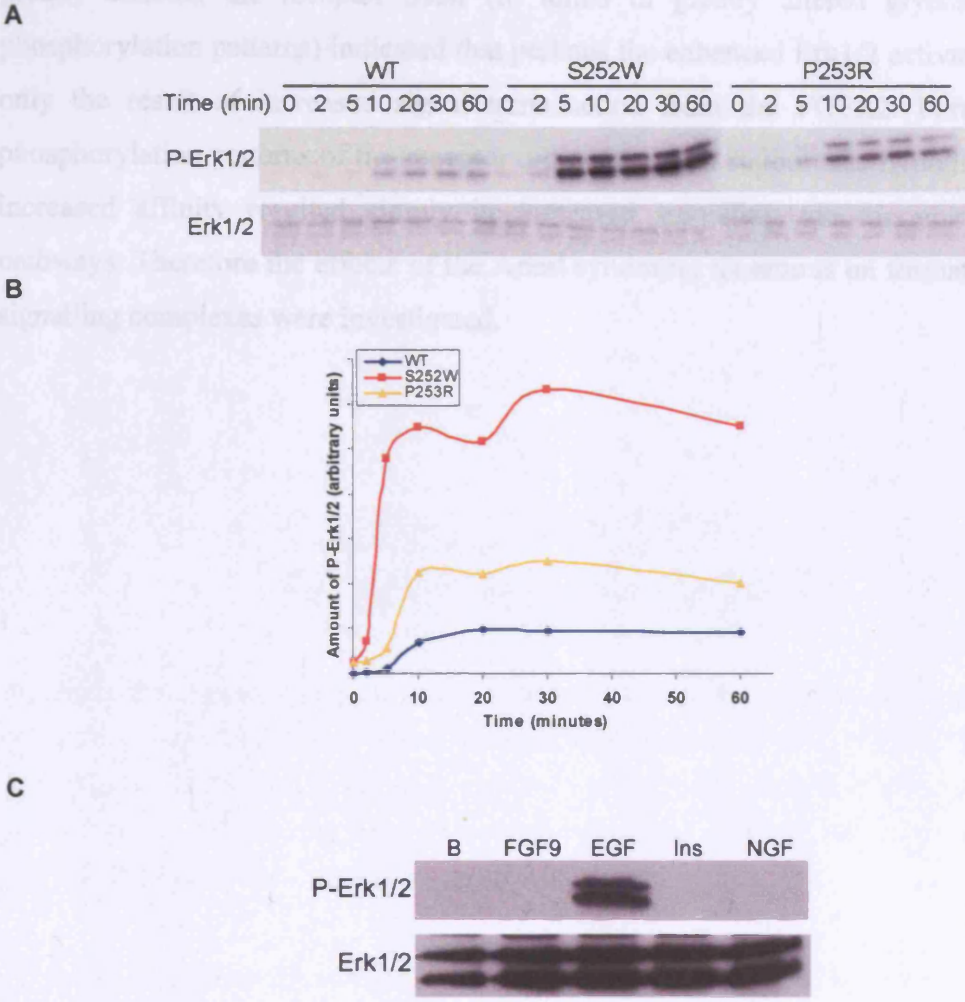


Figure 5.1: The Apert syndrome mutations lead to enhanced Erk1/2 activation HEK 293T cells were serum-starved overnight and stimulated with 10ng/ml FGF9 for various periods of time. 200µg of cell lysate were subjected to SDS-PAGE and immunoblotting with an anti-phospho-Erk1/2 antibody. To assess equal loading, blots were stripped and re-probed with an anti-Erk1/2 antibody (A). The amounts of phospho-Erk1/2 were assessed by densitometry and represented graphically for each time course experiment (B). The results shown are representative of three independent experiments. Untransfected HEK 293T cells were stimulated with 10ng/ml FGF9, EGF or NGF or 100µM insulin for ten minutes, lysed and subjected to SDS-PAGE and immunoblotting with an anti-phospho-Erk1/2 antibody. The blot was stripped and re-probed with an anti-Erk1/2 antibody (C).

5.2.2 The Apert syndrome mutations affect the overall intracellular phosphotyrosine content

One of the questions that arose from having observed the enhanced activation of the Erk1/2 pathway by the Apert mutant receptors, was whether this effect was simply due to the increased number of receptors activated as a result of their increased affinity for FGF ligand or whether it was a result of altered recruitment of adaptor proteins and signalling complex formation. The fact that both the S252W and the P253R mutations greatly affected the receptor itself (in terms of greatly altered glycosylation and phosphorylation patterns) indicated that perhaps the enhanced Erk1/2 activation was not only the result of increased signal transduction from the FGFR2. Particularly the phosphorylation patterns of the receptor did not seem to support the hypothesis that the increased affinity resulted simply in increased signalling via the normal FGFR2 pathways. Therefore the effects of the Apert syndrome mutations on formation of early signalling complexes were investigated.

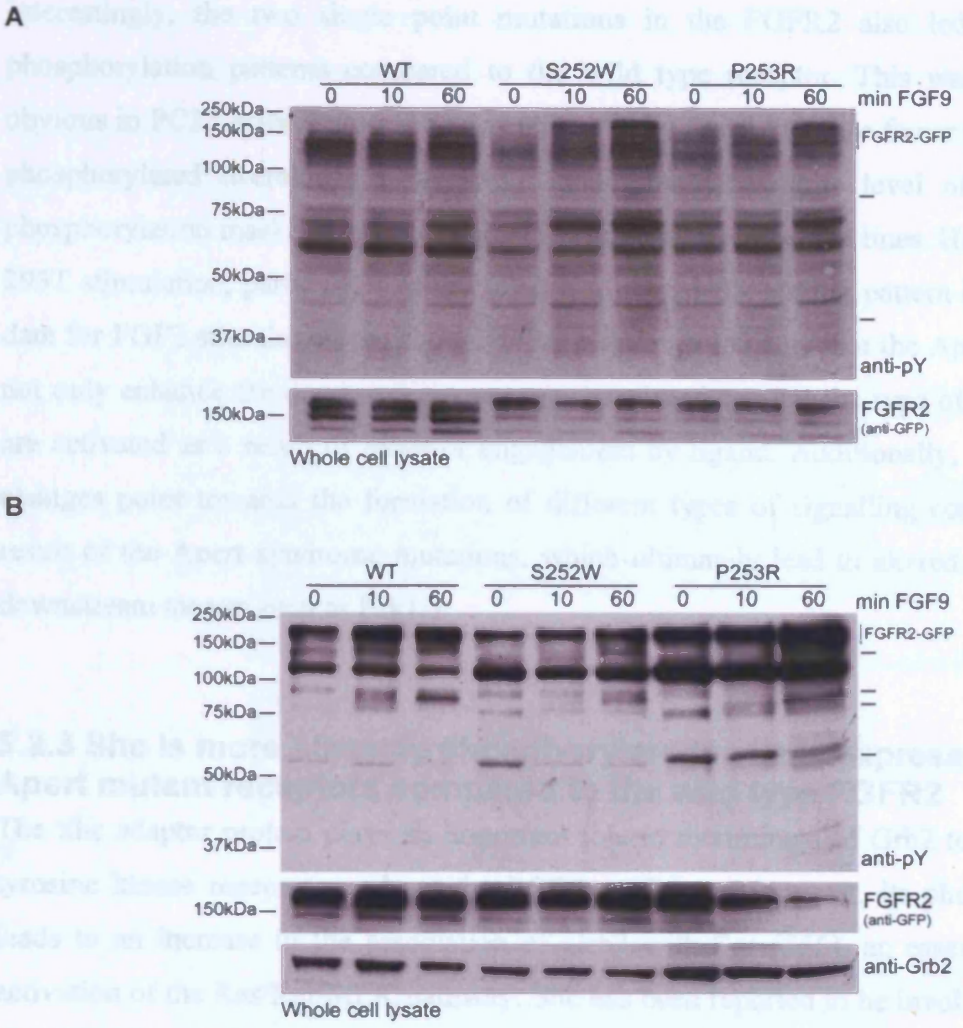


Figure 5.2: The Apert syndrome mutations affect total cellular tyrosine phosphorylation HEK 293T (A) or PC12 cells (B) were serum-starved overnight and stimulated with 10ng/ml FGF9. Cells were lysed and 200μg/ml of the cells lysates were subjected to SDS-PAGE and immunoblotting with an anti-phosphotyrosine antibody. The western blots were stripped and re-probed with an anti-GFP and/or an anti-Grb2 antibody to assess equal expression levels and gel loading.

As an initial indication of the effects of the changes in the Apert syndrome receptors on intracellular signalling, the changes in total cellular tyrosine phosphorylation were assessed (Figure 5.2). As expected from the enhanced Erk1/2 phosphorylation in response to activation of the S252W or P253R receptors, some proteins were more strongly phosphorylated in cells expressing the mutant receptors. Although one could propose that this trend is due to the increased affinity of the mutant receptors for ligand, the phosphorylation of the S252W and P253R receptors did not follow this enhanced pattern compared to the wild type receptor as described in Chapter 4. Thus other mechanisms must affect the way in which these proteins are recruited to the receptor to lead to their increased phosphorylation.

Interestingly, the two single point mutations in the FGFR2 also led to different phosphorylation patterns compared to the wild type receptor. This was particularly obvious in PC12 cells (Figure 5.2B: as indicated by lines), because fewer proteins were phosphorylated overall. In HEK 293T cells, the fairly large level of background phosphorylation masked certain differences between the three cell lines. However, HEK 293T stimulation, particularly with FGF2, also revealed a similar pattern (Figure 5.2A, data for FGF2 stimulation not shown). These findings indicate that the Apert mutations not only enhance the downstream response, but that they alter the type of proteins that are activated as a result of receptor engagement by ligand. Additionally, the observed changes point towards the formation of different types of signalling complexes as a result of the Apert syndrome mutations, which ultimately lead to altered activation of downstream targets such as Erk1/2.

5.2.3 Shc is more strongly phosphorylated in cells expressing the Apert mutant receptors compared to the wild type FGFR2

The Shc adaptor protein plays an important role in recruitment of Grb2 to a variety of tyrosine kinase receptors such as the EGFR or TrkA. Moreover, its phosphorylation leads to an increase in the association of Grb2 with Sos [235], an essential step for activation of the Ras/Raf/MEK pathway. Shc has been reported to be involved in FGFR signalling, but its exact role has not been elucidated [103-105]. On the phosphotyrosine blots (Figure 5.2), bands around 50-55kDa could be identified that were more strongly tyrosine phosphorylated in cells expressing the mutant receptors and were identified as Shc. This observation prompted an investigation into whether Shc was involved in signalling from the FGFR2 and whether the Apert mutations affected its role and recruitment downstream of the receptor.

To investigate whether the Apert syndrome mutations in FGFR2 have an effect on Shc phosphorylation, Shc was immunoprecipitated from cells expressing the wild type, S252W or P253R FGFR2 (Figure 5.3). In both HEK 293T and PC12 cells, there were remarkable differences in Shc phosphorylation, depending on which receptor was expressed. In cells expressing the wild type receptor, Shc was phosphorylated upon stimulation in PC12 cells. In HEK 293T cells, the basal level of phosphorylation was already quite high, which may be due to the fact that these cells are highly transformed and therefore the level of tyrosine phosphorylated proteins is generally high even in serum-starved, unstimulated cells. Since the overall effects of the Apert syndrome

mutations described so far were similar in both PC12 and HEK 293T cells, this basal Shc phosphorylation did not seem to interfere with general signal generation. By 30-60 minutes of stimulation, Shc phosphorylation decreased in response to wild type FGFR2 activation (Figure 5.3A and B: lane 3).

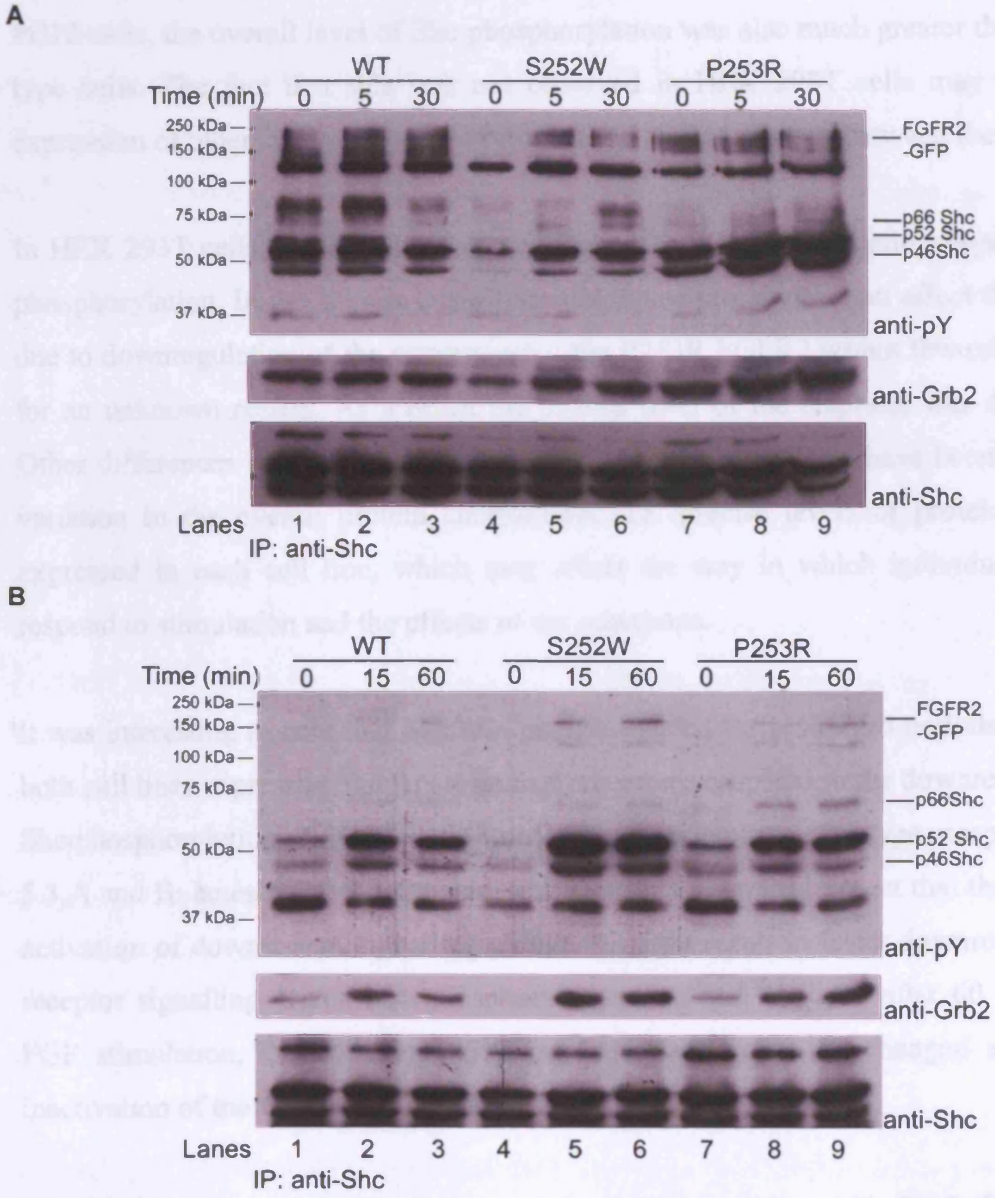


Figure 5.3: Shc immunoprecipitation reveals differences in Shc phosphorylation as a result of Apert syndrome mutations HEK 293T (A) or PC12 cells (B) expressing each of the three receptor constructs were serum-starved overnight and stimulated with 10ng/ml FGF9 as indicated. The stimulated time points differ between the two experiments as indicated. 3mg of cell lysate were subjected to immunoprecipitation with an anti-Shc antibody and subsequent immunoblotting using an anti-phosphotyrosine antibody. Blots were stripped and re-probed for Grb2 and Shc using appropriate antibodies.

The Shc phosphorylation in S252W cells mirrored that of the FGFR2 (refer to Figure 4.2): Shc phosphorylation was very low in unstimulated cells but increased largely upon addition of FGF9. In both cell lines expression of the S252W mutant receptor negatively regulated Shc phosphorylation in unstimulated cells, meaning that the levels were reduced compared to those in cells expressing the wild type or P253R receptors. In PC12 cells, the overall level of Shc phosphorylation was also much greater than in wild type cells. The fact that this was not observed in HEK 293T cells may be due to expression of other cell type specific proteins and slight variation between the cell lines.

In HEK 293T cells, the P253R receptor induced very high, stimulation-dependent Shc phosphorylation. In PC12 cells this effect was not as pronounced, an effect that may be due to downregulation of the expression of the P253R FGFR2 within few cell passages for an unknown reason. As a result the overall level of the response was diminished. Other differences between HEK 293T and PC12 cells might also have been caused by variation in the overall protein complement (i.e. precise levels of proteins present) expressed in each cell line, which may affect the way in which individual proteins respond to stimulation and the effects of the mutations.

It was interesting to note that Shc was phosphorylated for prolonged periods of time in both cell lines expressing the Apert mutant receptors compared to the downregulation of Shc phosphorylation after to 30-60 minutes stimulation of the wild type receptor (Figure 5.3 A and B: lanes 6 and 9 compared with lane 3). One would expect that the enhanced activation of downstream signalling pathways might result in faster downregulation of receptor signalling. Since Shc phosphorylation was still elevated after 60 minutes of FGF stimulation, the downregulation mechanisms seem to be changed and normal inactivation of the various pathways does not occur.

The P253R mutation resulted in increased association of Shc with Grb2 (Figure 5.3A and B: middle panels lanes 8-9 compared to lanes 2-3). The S252W mutation only resulted in increased Shc-Grb2 association in PC12 cells, which is in agreement with the increased phosphorylation level in these cells. The changes in association with Grb2 indicate that Shc is involved in FGFR2 signalling and activation of the Erk1/2 pathway downstream of this receptor. In addition to increased association of Shc with Grb2 in cells expressing the Apert syndrome mutant receptors, an unidentified protein of approximately 85kDa (p85) was found to be differentially associated with Shc in HEK

293T cells depending on which receptor was expressed. This tyrosine phosphorylated protein was primarily associated with Shc in cells expressing the wild type receptor (Figure 5.3 A: lanes 1-3). Concomitant with the downregulation of Shc phosphorylation, less of this protein was observed on the anti-phosphotyrosine blot following prolonged FGF9 stimulation (Figure 5.3: lane 3), which may represent decreased association with Shc or simply decreased phosphorylation of the p85. This protein was only minimally associated with Shc (or minimally phosphorylated) in cells expressing the S252W or the P253R receptors. These observations indicate that Shc is not only differentially phosphorylated in cells expressing either of the Apert mutant compared to the wild type FGFR2, but that it is also recruited to a different signalling complex.

5.2.4 Use of RFP-tagged Shc to investigate the interaction with FGFR2

The Shc immunoprecipitation experiment indicated that Shc is involved in signalling downstream of the FGFR2 and that it is involved in a different signalling complex in cells expressing the S252W or P253R mutant receptors compared to the wild type FGFR2. Based on these observations, it was chosen to investigate the co-localisation behaviour of the FGFR2 and Shc in HEK 293T cells, to analyse whether the Apert mutations affect the recruitment of Shc to the FGFR2 signalling complex. Before studies were carried out, it needed to be assessed whether the RFP-tagged construct was fully functional and could be phosphorylated like endogenous Shc to ensure that normal signalling behaviour was not affected by the addition of the RFP-tag.

Full-length C-terminally RFP-tagged Shc and Shc in which all three phosphorylatable tyrosine residues (Y239, Y240 and Y317) were mutated to phenylalanine (Shc3F) were transiently transfected into cells expressing the wild type FGFR2. Immunoprecipitation with an anti-Shc antibody was carried out, because the anti-RFP antibody was not very specific and resulted in isolation of a lot of non-specific proteins. The RFP-tagged Shc construct was fully phosphorylated (Figure 5.4) whereas the Shc3F construct could not be phosphorylated. This confirmed that the RFP-tag did not interfere with Shc phosphorylation by the receptor. The fully functional Shc-RFP construct could subsequently be employed in co-localisation studies.

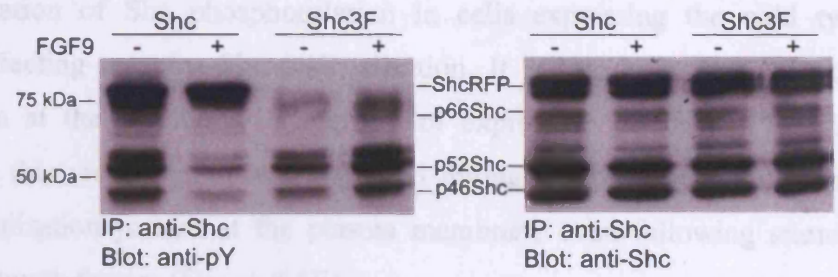


Figure 5.4: Establishment of a functional RFP-tagged Shc construct HEK 293T cells expressing the wild type FGFR2 were transiently transfected with the Shc-RFP or Shc3F-RFP constructs using calcium phosphate precipitation. Cells were serum-starved overnight, stimulated with 10ng/ml FGF9 for 15 minutes and lysed. Cell lysates were subjected to immunoprecipitation with an anti-Shc antibody and subsequent immunoblotting with an anti-phosphotyrosine antibody. The western blot was stripped and re-probed for total Shc using an appropriate antibody.

5.2.5 Effect of Apert mutations on co-localisation of the FGFR2 with Shc

To investigate the involvement of Shc in FGFR2 signalling and possible changes that are implemented by the Apert syndrome mutations, C-terminally RFP-tagged Shc was transiently co-transfected into HEK 293T cells stably expressing one of the three different FGFR2-GFP constructs respectively. The cells were stimulated for various time periods and analysed by confocal microscopy. Although the focal plane is not exactly the same for each cell presented, a mid-section was always chosen to avoid distortion of results by changes in protein localisation introduced by cell adhesion to the glass slide.

Figure 5.5A shows representative images obtained for cells co-expressing the wild type FGFR2 and Shc. Although Shc was partially localised at the membrane in unstimulated cells, its membrane localisation increased upon stimulation of cells with FGF9 (Figure 5.5A: five minutes). Following 15 minutes of exposure to FGF9, Shc was temporarily localised more diffusely throughout the cell, although it still co-localised with the FGFR2 at the membrane. By 30 and 60 minutes of stimulation, Shc almost exclusively co-localised with the wild type receptor (refer to graphs indicating the intensities of GFP (green) and RFP (red) along the arbitrarily drawn line through the respective cell). This co-localisation occurred along the plasma membrane and inside vesicles after longer exposure to growth factor (Figure 5.5A: 30 and 60 minutes). Although Shc phosphorylation started to decrease by 30 minutes of FGF9 stimulation (Figure 5.3A), its co-localisation with the receptor did not change. Internalisation may cause

downregulation of Shc phosphorylation in cells expressing the wild type FGFR2 without affecting receptor Shc co-localisation. It is also important to note, that Shc localisation at the membrane is a result of expression of the FGFR2. In cells not expressing this receptor, Shc was localised throughout the cell and did not portray a strong localisation pattern at the plasma membrane even following stimulation with different growth factors (Figure 5.5E).

The P253R FGFR2 and Shc portrayed a co-localisation pattern that was very similar to that of the wild type receptor and Shc (Figure 5.5C). However, in slight contrast, there was hardly any co-localisation of Shc and the P253R receptor in unstimulated cells (Figure 5.5C: panels f and k) whereas in cells expressing the wild type receptor, Shc was already localised near the membrane without exposure of the cells to FGF9 (Figure 5.5A: panels f and k). Upon stimulation, the co-localisation of Shc and the P253R FGFR2 increased with longer exposure of cells to FGF9. By 30 to 60 minutes of stimulation, almost all Shc co-localised with the receptor. The P253R receptor is unable to undergo endocytosis ([224], in submission and as observed in Figure 5.5). This was reflected in the lack of co-localisation of Shc with the P253R FGFR2 on any vesicular structures as was observed for the wild type receptor after prolonged exposure to FGF9 (compare Figure 5.5A and C: panels d and e). In the case of the P253R receptor, co-localisation with Shc took place exclusively at the plasma membrane. As a result, Shc, even though partly localised diffusely throughout the cytoplasm, was not present in any intracellular vesicles in these cells. The lack of endocytosis of this receptor and thus of Shc could be the regulator of the enhanced levels of Shc phosphorylation observed in cells expressing this mutant receptor (Figure 5.3A).

Cells expressing the S252W FGFR2 displayed an altogether different pattern of Shc co-localisation with the receptor (Figure 5.5B). Although Shc portrayed a low amount of co-localisation with the receptor, this occurred largely in the ER/Golgi, where both proteins were synthesized and thus were found in the same location. However, Shc was only minimally localised at the plasma membrane in S252W cell. This did not change throughout the time course of stimulation up until 60 minutes. Strikingly however, 60 minutes post-stimulation, Shc co-localised with the S252W FGFR2 in intracellular vesicles (Figure 5.5B: panel o). It is unclear why this occurs, but it portrays a subtle yet important difference from the pattern observed in cells expressing either the wild type or the P253R FGFR2. In the case of the S252W receptor, internalisation of the receptor

and co-localisation with Shc did not result in downregulation of Shc phosphorylation as was the case for the wild type receptor.

Thus overall, the Apert syndrome mutations affected Shc phosphorylation as well as altering its localisation pattern. These aspects could play an important role in determining the ultimate downstream response created by each respective receptor. The altered recruitment of Shc is likely to be transmitted to downstream signalling pathways and may be an important factor in changes in multiprotein complex formation after receptor activation.

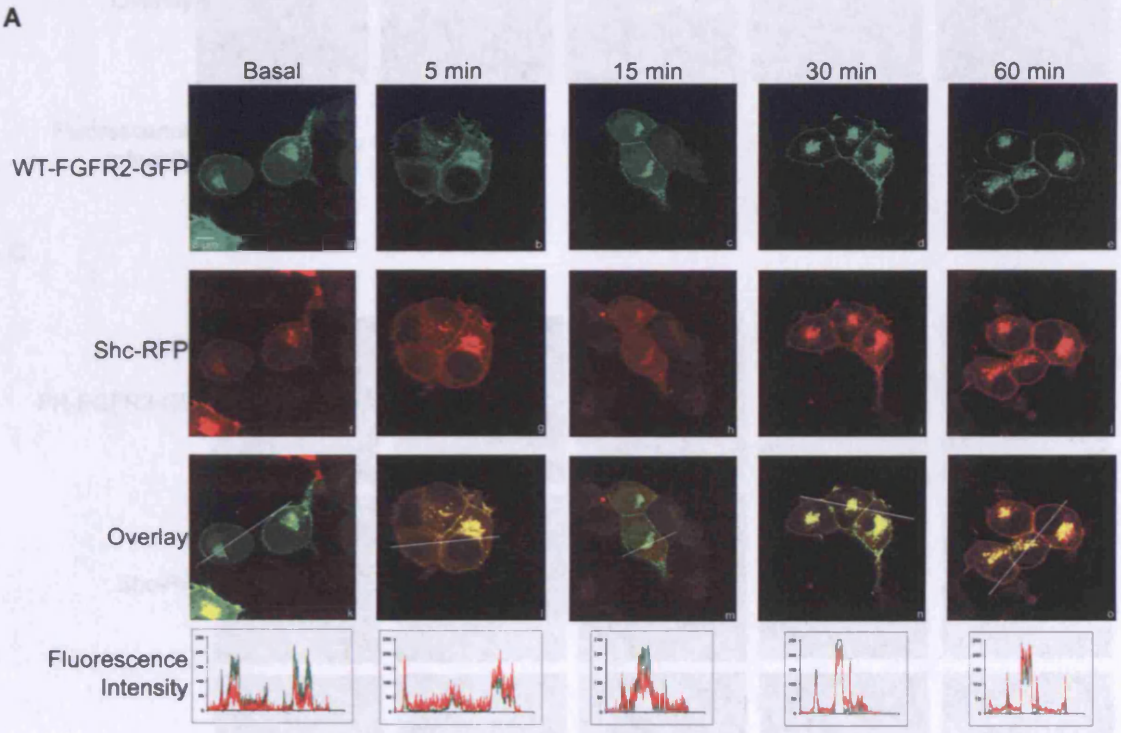
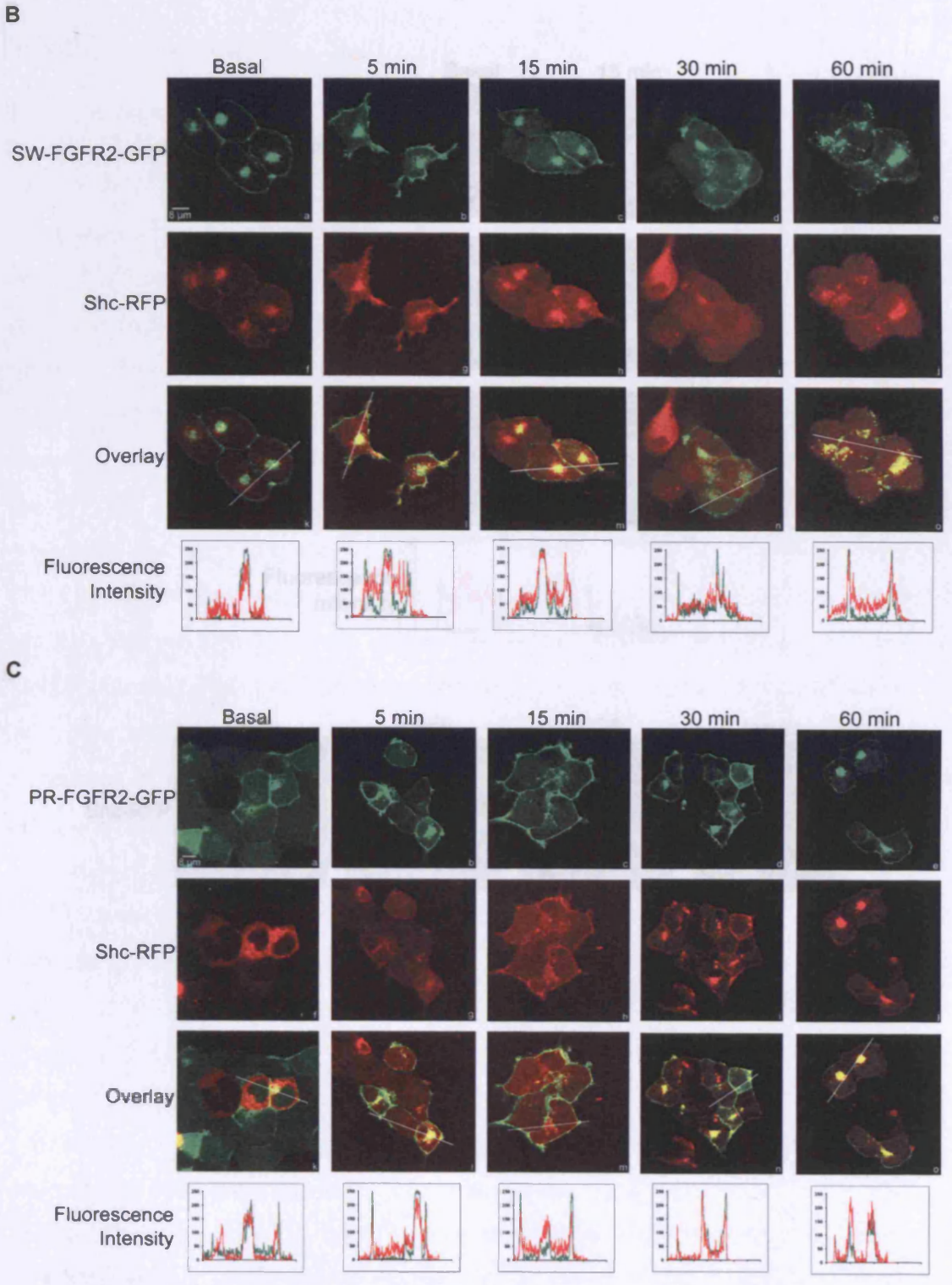


Figure 5.5: Co-localisation of Shc with the wild type, S252W and P253R FGFR2 HEK 293T cells expressing the wild type (A), S252W (B, page 171) or P253R (C, page 171) FGFR2 or HEK 293T cells alone (E, page 172) were transiently transfected with Shc-RFP using Lipofectamine 2000. HEK 293T cells expressing the WT FGFR2 were transfected with RFP alone. Cells were seeded on glass coverslips, serum-starved overnight and stimulated with 10ng/ml FGF9 for various time periods as indicated or additionally with 10ng/ml FGF2, EGF, or 100μM insulin for five minutes (D, page 172). Cells were fixed in 4% paraformaldehyde, washed extensively in PBS and analysed using a Leica SP2 confocal microscopy system. Images shown are representative of at least 10 images obtained. The relative fluorescence intensities of GFP and RFP along an arbitrarily drawn line are represented graphically in green and red respectively.


B
Basal
5 min
15 min
30 min
60 min
SW-FGFR2-GFP
Shc-RFP
Overlay
Fluorescence Intensity
C
Basal
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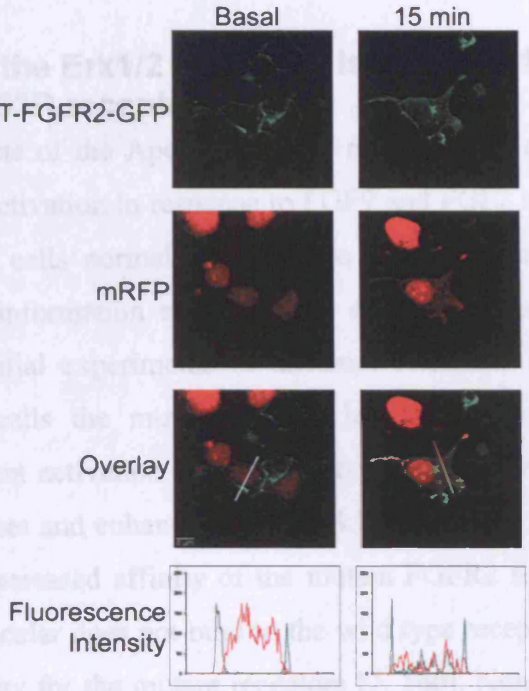
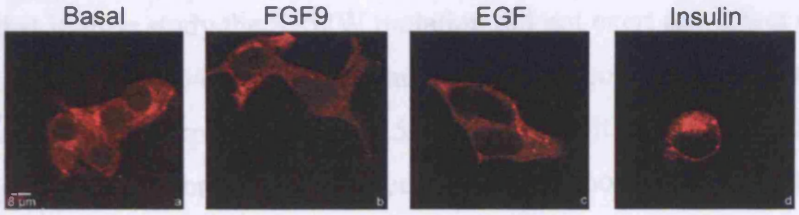
30 min
60 min
PR-FGFR2-GFP
Shc-RFP
Overlay
Fluorescence Intensity

D


Basal
15 min
WT-FGFR2-GFP
mRFP
Overlay
Fluorescence Intensity

E


Basal
FGF9
EGF
Insulin
Shc-RFP

5.3 Discussion

5.3.1 Activation of the Erk1/2 pathways is enhanced downstream of the S252W and P253R receptors

One of the major effects of the Apert syndrome mutations on downstream signalling was enhanced Erk1/2 activation in response to FGF9 and FGF2 stimulation. Since both HEK 293T and PC12 cells normally respond to FGFR stimulation with prolonged Erk1/2 activation, no information regarding the effect on signal longevity could be obtained. However, initial experiments in different cell lines indicated that in ROS 17/2.8 osteosarcoma cells the mutations also led to prolonged Erk1/2 activation compared to its transient activation by the wild type FGFR2 (Z. Ahmed, unpublished results). The earlier onset and enhancement of Erk1/2 signalling correlate well with the previously described increased affinity of the mutant FGFR2 for various FGF ligands [7, 161]. FGF9 in particular does not bind to the wild type receptor strongly, but shows greatly enhanced affinity for the mutant receptors [7, 160]. Nonetheless, an increase in Erk1/2 phosphorylation has not been reported previously. On the contrary, Mansukhani *et al.* reported that in their study the S252W mutation did not exert any effect on Erk1/2 signalling in osteoblasts [164]. However, another study correlated an increase in mitogenic activity in cells expressing the S252W FGFR2 with increased affinity for various FGF ligands, and reported an increased mitogenic reponse to FGF9 stimulation in the presence of the S252W mutation [160]. Although mitogenicity is not a direct measure of Erk1/2 activation, this pathway certainly contributes greatly to this cellular process. The observed increase in Erk1/2 activation in cells expressing the Apert mutant receptors presented in this work are somewhat in agreement with the previously described increased in mitogenic response. However, maximal stimulation of the wild type receptor by addition of excess ligand does not lead to activation of Erk1/2 to a level comparable with that induced by activation of the S252W or P253R receptors. This indicates that the enhanced Erk1/2 phosphorylation levels downstream of the mutant receptors are not solely due to engagement of more receptors because of increased ligand affinity but that additional factors such as alterations in the receptor and its signalling pathways must contribute to the observed phenomenon. Thus although the increased affinity of the mutant receptors for FGF may play a role in enhanced Erk1/2 signalling, the data presented in Chapter 4 also indicate that various other changes in the FGFR2 underlie this differential activation of major signalling pathway. Since various signals can converge on Erk1/2 activation, more complex changes in the early signalling

events downstream of the mutant receptors may be responsible for the increased phosphorylation of Erk1/2.

5.3.2 Enhanced Erk1/2 activation does not result in increased FGFR2 signal downregulation

It was interesting to note that the increased activation of the Erk1/2 pathway did not result in increased downregulation of the signal. Negative feedback via phosphorylation of Sos and FRS2 by Erk1/2 takes place downstream of the FGFR normally and is important in regulating the signals emanated from the activated receptor (reviewed in [92]). Interestingly, the enhanced activation of Erk1/2 did not result in increased signal downregulation. Particularly the observation that the mutant receptors not only result in enhanced but also prolonged activation of the Erk1/2 pathway in ROS 17/2.8 cells indicates that their downregulation must be impaired somehow. This effect becomes particularly apparent when assessing the internalisation behaviour of the S252W and P253R receptors. Both receptors portrayed altered endocytosis following FGF9 stimulation and were retained at the plasma membrane to a greater extent (i.e. did not undergo the same level of endocytosis as the wild type receptor) ([224], in submission and Figure 5.5). In addition to the numerous effects of the Apert mutations on the FGFR2 itself, these observations indicate that the enhanced Erk1/2 activation may be the result of mechanisms other than enhancement of the normal signalling pathways. These findings therefore led to the investigation of whether the effects on Erk1/2 signalling were simply due to 'upregulation' of the normal signalling pathways from the FGFR2 due to prolonged receptor engagement or whether the mutations actually affect protein recruitment to the receptor and thereby alter signalling pathways.

5.3.3 The Apert syndrome mutations affect the integrity of protein recruitment and phosphorylation downstream of the FGFR2

Having observed various effects of the Apert syndrome mutations on the FGFR2 itself as well as on one of the main downstream signalling pathways it was chosen to address the question whether the Apert syndrome mutations affect the integrity of FGFR2 signalling by altering protein recruitment to the receptor. Differences in protein recruitment to the mutant receptors could be responsible for the visible increase in Erk1/2 phosphorylation since several 'pathways' converge on this kinase and therefore

an increase in its activation could also reflect changes in protein recruitment to the activated receptor.

Analysis of receptor phosphorylation (Figures 5.2 and 4.5) and phosphorylation of various cellular proteins by western blotting with an anti-phosphotyrosine antibody revealed that the changes introduced in total cellular phosphotyrosine content by the two Apert mutations did not necessarily mirror the different receptor phosphorylation patterns. Whereas some proteins were more strongly tyrosine phosphorylated in cells expressing one of the Apert mutant receptors, others were less phosphorylated or portrayed variations in time-dependent phosphorylation changes (Figure 5.2). Overall, both mutant receptors led to (i) increased phosphorylation of some proteins that were also phosphorylated in cells expressing the wild type receptor and (ii) phosphorylation of proteins that were not phosphorylated in wild type cells. These data indicate that the S252W and P253R mutations not only manifest the Apert syndrome phenotype as a result of enhanced signalling, but also cause the activation of different/altered signalling pathways.

The observation that the Apert syndrome mutations do not simply upregulate the normal signal induced by the FGFR2 upon stimulation, but activate different signalling proteins/pathways, initiated the investigation of the effects of the Apert syndrome mutation on spatio-temporal recruitment of adaptor proteins to the receptor. Analysis of the localisation of both FRS2 and Grb2 in response to activation of the Apert mutant receptors compared to the wild type receptor revealed that both proteins were associated very differently with each of the receptors in stimulated and unstimulated states ([224], in submission and Z. Ahmed, unpublished results). These findings indicated more precisely, that the presence of the Apert syndrome mutations indeed affects the integrity of the 'normal' protein complex assembly and leads to altered recruitment of signalling proteins to the FGFR2.

5.3.4 The implications of altered Shc recruitment on FGFR2 downstream signalling

The altered recruitment of Shc to the FGFR2 was assessed in this work, since Shc has been implicated with a role in FGFR signalling [100, 103-105, 278]. Analysis of Shc phosphorylation in response to FGFR2 activation revealed that Shc is phosphorylated

downstream of the FGFR2. Interestingly the level of phosphorylation was enhanced by both the S252W and the P253R mutations (Figure 5.3). Furthermore, whereas Shc phosphorylation declined with prolonged activation of the wild type receptor, the level of Shc phosphorylation increased further with longer S252W or P253R activation. This indicates that significant changes occur in the overall signalling pathways emanated from the FGFR2 in the presence of these two mutations. The observed increase in phosphorylation resulted in increased Grb2 binding and precipitation although slight cellular differences were observed between HEK 293T and PC12 cells (Figure 5.3A and B: middle panels). These findings demonstrate a role for Shc in FGFR2 signal transduction and also implement it with a possible role in the manifestation of Apert syndrome phenotypes.

In addition to increased phosphorylation, Shc portrayed altered recruitment to the FGFR2 in the presence of the Apert syndrome mutations (Figure 5.5). Interestingly, differences in Shc recruitment were also observed between the two mutant receptors themselves. In P253R cells, the recruitment of Shc to the receptor was not significantly altered compared to the wild type receptor. In both wild type and P253R expressing cells Shc co-localised with the receptor at the plasma membrane as well as being found in the ER/Golgi compartments where both proteins are being synthesised (Figure 5.5A and C). Thus in the case of the P253R receptor, the spatio-temporal recruitment of Shc to the receptor was not greatly affected, but it was significantly more phosphorylated. In addition to the changes the P253R mutation inflicts on FRS2 recruitment and the reduced endocytic behaviour of this receptor ([224], in submission), the increased Shc phosphorylation would be expected to contribute further to the increased Erk1/2 phosphorylation by recruitment of a greater amount of the Grb2/Sos complex to the plasma membrane. It was recently shown that by abolishing the ability of FRS2 to bind to FGFR2, the phenotype of Crouzon syndrome (as well as the enhanced Erk1/2 activation) could be reversed in mice [279]. These findings indicate that FRS2 is mainly responsible for mediating the effects of the Crouzon mutations, and a similar scenario may be the case in Apert syndrome. We have additionally, shown that the recruitment and interaction of FRS2 with the FGFR2 is greatly altered in the presence of the Apert syndrome mutations, which seems to indicate that this may be one of the main mechanisms by which Apert syndrome is manifested ([224], in submission). However, the increased Shc phosphorylation in cells expressing the P253R mutant receptor would

also be expected to contribute significantly to the enhanced activation of the Erk1/2 pathway.

In comparison to cells expressing the wild type and the P253R receptors, cells expressing the S252W receptor portrayed a considerably altered Shc localisation pattern (Figure 5.5B). Whereas Shc was highly membrane localised with both the wild type and the P253R FGFR2, in the presence of the S252W receptor it was found to be located more diffusely throughout the cell. Although co-localisation with the receptor occurred to a small extent at the membrane, a large amount of Shc was also localised in regions from which the receptor was excluded (Figure 5.5B). Interestingly, after 60 minutes of exposure of cells to FGF9, Shc was found to be highly localised with the receptor in intracellular vesicles. This indicates that indeed Shc is differentially recruited to the S252W FGFR2 which represents the formation of different signalling complexes compared to those assembled upon activation of the wild type FGFR2. This may then greatly affect the activation of downstream targets such as Erk1/2. It is interesting to note that co-localisation of Shc with the S252W receptor after 60 minutes of FGF9 stimulation and with the P253R receptor occurs despite a lack of Shc association with the unidentified p85 that was found in immunoprecipitates from cells expressing the wild type FGFR2. This indicates the formation of altered signalling complexes as well as the fact that various factors must be involved in regulating Shc recruitment to the FGFR2.

Interestingly the absence of Shc co-localisation with the S252W FGFR2 did not coincide with a decreased, but rather an increased, level of Shc phosphorylation. This observation indicates that Shc may briefly be membrane localised to become phosphorylated but then redistributes to the cytoplasm. This hypothesis would be in agreement with the low levels of co-localisation observed in the earlier time points (Figure 5.5B: panels l-n). The cytoplasmic Shc is likely to affect signalling in a different way to the membrane bound Shc, since recruitment of Grb2/Sos at the membrane is essential for Ras activation [280]. Shc may therefore contribute significantly to the altered cellular behaviour that contributes to the manifestation of Apert syndrome in different ways in cells expressing the S252W mutant, whereas other proteins such as FRS2 play an important role in enhanced activation of the Erk1/2 signalling pathway.

Altogether, the S252W mutation was found to alter the cellular localisation of Shc in addition to its phosphorylation, whereas the P253R mutation did not significantly affect the co-localisation with the receptor. Firstly, this finding indicates that there are differences in early signalling events downstream of the S252W and the P253R receptors. This is particularly interesting in light of both mutations causing very similar phenotypes (i.e. craniosynostosis and syndactyly) in the whole organism. These differences further indicate that constant localisation of Shc at the membrane is not required for its phosphorylation. Secondly, the fact that Shc is phosphorylated more strongly and for prolonged periods in response to stimulation of either Apert mutant receptor compared to the wild type indicates that Shc may indeed play an important role in Erk1/2 activation downstream of the FGFR2. Although FRS2 is the main Grb2-recruiting component in FGFR signalling, mice lacking the FRS2 binding site on FGFR1 were able to activate Erk1/2 and undergo the somitogenesis and gastrulation stages of development normally [281], which indicates that other proteins, most likely Shc, are able to activate the Erk1/2 pathway to an extent at which development remains unaffected. Further studies would need to be carried out to quantitatively assess the possible changes in Grb2 recruitment by Shc and FRS2 in the presence of receptors containing the Apert syndrome mutations.

5.3.5 Effects of altered Shc recruitment and phosphorylation on signalling from the FGFR2

The main role of Shc in response to activation of growth factor receptors such as the EGFR, PDGFR or TrkA is recruitment of the Grb2/Sos complex whereby activation of the Ras/Raf/MEK/Erk1/2 is initiated. FRS2 has been proposed to be the main Grb2 recruiting protein in FGFR signalling [47], whereas the role of Shc has not been fully elucidated since phosphorylation by, but no interaction with, the FGFR1 has been described [104]. Nonetheless, Shc carries out an important function in FGFR signalling since it has been shown to be involved in FGFR-stimulated mitogenesis [282] and the Shc SH2 domain was found to block FGF1 induced Erk2 phosphorylation in *Xenopus laevis* oocytes expressing the *Pleurodeles* FGFR1 [283]. It was therefore interesting to note that Shc phosphorylation was both enhanced and prolonged in cells expressing the Apert syndrome mutations, since this indicates that alterations in Shc phosphorylation may play a role in the enhanced Erk1/2 phosphorylation observed in response to S252W or P253R FGFR2 stimulation. The increased amount of Grb2 co-precipitated with Shc

(Figure 5.3) also indicates that Shc may play a role in activation of the Erk1/2 pathway in general and particularly the enhanced activation of this pathway in response to mutant FGFR2 activation. It is interesting to note, however, that Shc is only minimally membrane localised in cells expressing the S252W receptor, which indicates that increased Shc phosphorylation must also be involved in other functions downstream of the FGFR2 that do not require membrane localisation.

Apart from the FGFR2, relatively few Shc interacting phospho-proteins were observed in the Shc immunoprecipitation experiments (Figure 5.3). The approximately 120kDa phospho-protein that co-precipitated with Shc in HEK 293T cells (and could also be identified upon longer exposure in immunoprecipitates from PC12 cells, data not shown) could play a role in mediating functions other than Grb2 recruitment in response to FGFR stimulation. A 37-40kDa protein was also co-precipitated with Shc from both HEK 293T and PC12 cells, and could be the p40 protein that binds to the pY239/pY240 site of Shc described by van der Geer *et al.* [198]. Most interestingly, an approximately 85Da protein was co-precipitated (from HEK 293T cells only), and was primarily found in immunoprecipitates from cells expressing the wild type FGFR2. This indicates that Shc is indeed involved in forming different signalling complexes upon receptor activation in the presence of the mutations. This protein could be 80K-H, a tyrosine phosphorylated protein that has been shown to be involved in signalling from the FGFR1 and 3 [106, 284, 285]. 80K-H has been shown to be in complex with Grb2 and Sos, but has not been shown to interact with Shc directly, although formation of a ternary complex cannot be excluded. Alternatively the phospho-protein could be a 85kDa serine kinase has been shown to be phosphorylated by and associated with the FGFR4 but not the FGFR1 [286-288]. The role of this unidentified p85 is unclear and only identification of this protein (and other Shc interacting partners) can indicate the other signalling pathways that Shc is involved in downstream of the FGFR2 and how the Apert mutations affect them. In conjunction with the various other proteins that were found to be differentially phosphorylated in cells expressing the Apert mutant receptors (Figure 5.1) the co-precipitation of these unidentified proteins (particularly p85) suggests the possibility that Shc could be part of an altered signalling complex after activation of the mutant receptors compared to the wild type receptors. Identification of the proteins co-precipitated with Shc would reveal the pathways other than Erk1/2 activation that Shc is involved in following FGF stimulation. Additionally, their

identification would elucidate how these interactions are affected by altered Shc phosphorylation and recruitment in the presence of the Apert mutations.

5.3.6 The manifestation of Apert syndrome involves altered protein recruitment to the FGFR2

Mutations in the FGFR, including the Apert mutations in the FGFR2, have been previously described as gain-of-function when acting through various mechanisms such as constitutive activation, illegitimate splicing or, as in the case of the S252W and P253R mutations, through increased ligand binding affinity and illegitimate ligand binding [173, 174]. The term 'gain-of-function' implies that the Apert mutations simply result in enhanced activation of the regular signalling pathways as a result of a greater number of receptors being engaged by ligand at any given time.

On the contrary, the results presented in this work, in conjunction with other findings made in the laboratory, indicate that Apert syndrome is manifested by altered recruitment of signalling proteins and formation of different signalling complexes rather than simply the increased activation of regular FGFR2 signalling pathways. The differences in Shc phosphorylation and localisation in cells expressing the S252W or P253R receptors as well as changes in recruitment of FRS2 and Grb2 ([224], in submission and Z. Ahmed, unpublished results) indicate that altered protein recruitment to the FGFR2 forms the basis for Apert syndrome. In conjunction with the changes observed in total intracellular tyrosine phosphorylation, these findings indicate that the term gain-of-function is perhaps inappropriate, since additional mechanisms such as altered formation of multiprotein complexes contribute to the upregulation of downstream pathways such as Erk1/2 and hence the manifestation of Apert syndrome. In vivo, the alteration of receptor specificity for ligand recognition is thought to be an important determinant of Apert syndrome. However, the intracellular changes described herein clearly show that Apert syndrome is not merely the result of 'incorrect' activation of FGFR2 signalling by paracrine signalling (reviewed in [149]), but that the mutations also greatly affect the integrity of intracellular signalling. This is important for the understanding of the molecular basis of Apert syndrome, because such changes may result in activation of different pathways that would normally not be initiated downstream of the FGFR2 and may thereby result in the phenotypic symptoms of Apert syndrome. This study investigated the effects of changes in multiprotein assembly

downstream of the Apert mutant receptors on activation of the Erk1/2 kinase. However, further investigation of the effects on pathways such as the PI3K and PLCγ pathways may reveal the overall extent to which changes in protein complex formation as a result of the two extracelluar mutations affect signal generation downstream of the FGFR2. Altogether, the findings presented in this chapter indicate the presence of an additional layer of complexity to the way in which the S252W and P253R mutations result in Apert syndrome.

It is also interesting to note that the two Apert mutant receptors do not employ the same mechanism to achieve similar overall cellular outcomes such as enhanced Erk1/2 activation. This was revealed by the difference in recruitment of Shc (as well as Grb2 and FRS2 as assessed in separate studies ([224], in submission and Z. Ahmed, unpublished results) to the S252W or the P253R receptors. This indicates an overall change in the assembly of protein complexes in response to receptor activation and very different effects depending on the exact mutation present. It is possible that the differences in the intracellular effects caused by either the S252W or the P253R mutations are important in mediating the phenotypic differences observed in terms of the severity of syndactyly and craniofacial abnormalities [264]. These observations are interesting, because they show that individual mutations in the extracellular domains of tyrosine kinase receptors such as the FGFR2 are able to introduce very pronounced and unique effects on signalling complex formation. This significantly advances the general understanding of how mutations affect signal transduction by resulting in the formation of entirely different protein-protein interactions intracellularly, which then affects various cellular pathways and later the specificity of signalling from a given receptor. Additionally, it was shown that individually different cellular mechanisms can underlie the overall gain-of-function properties of the Apert mutant receptors.

5.3.7 Altered protein recruitment to the S252W and P253R receptors may be the result of the combined changes in the FGFR2

To date it is not clear exactly how the altered protein recruitment to either of the Apert mutant receptors is mediated. Receptor phosphorylation would be one of the main factors dictating which proteins are recruited and when, and is therefore a major determinant of multiprotein complex assembly. However, replacing all phosphorylation sites on FGFR1 with phenylalanine, with the exception of Y653 and Y654 whose

phosphorylation is required for kinase activation, neither affected the phosphorylation levels of Shc, FRS2 or Erk1/2 kinase, nor the Sos gel shift in response to FGF stimulation [103]. Mitogenesis or PC12 cell differentiation in response to FGF were also not significantly affected by the absence of these phosphorylation sites [103]. These data would therefore indicate that receptor phosphorylation is not the single most important component in protein recruitment to, and signal transduction from the FGFR2. Only mutation of FGFR2 tyrosine residues alone and in various combinations may indicate whether phosphorylation of individual residues is an important determinant of the differential recruitment of adaptor proteins. Further, it would also be interesting to elucidate whether stoichiometric differences in phosphorylation of various tyrosine residues in the presence of the Apert syndrome mutations are a factor affecting the changes in protein recruitment observed.

An important factor that has to be taken into account when analysing the altered intracellular signalling events that occur in response to activation of receptors containing the S252W or the P253R mutations is glycosylation. Correct receptor glycosylation has been shown to be important not only in receptor synthesis, quality control (reviewed in [257]) and localisation to the plasma membrane [258, 259], but in the case of the EGFR has also been shown to be an important factor in preventing premature endocytosis [276]. Thus as described in Chapter 4, the altered glycosylation pattern of the mutant receptors may play a role in the differences in receptor internalisation observed. Changes in endocytic behaviour may consequently affect protein recruitment to the receptor and thereby affect the signalling events downstream of the Apert mutant receptors. Additionally, changes in FGFR2 glycosylation may affect both ligand-dependent and independent dimerisation (refer to Chapter 4), which may in turn affect receptor phosphorylation and the availability of binding sites for various signalling proteins and thereby contribute to the formation of altered signalling complexes downstream of the mutant receptors compared to the wild type FGFR2.

Although the exact mechanism by which the S252W and P253R mutations result in altered protein recruitment to and complex assembly downstream of the FGFR2 is not clear, it seems that the various changes they incur on the FGFR2 play an important role in causing the changes. Changes in receptor glycosylation, phosphorylation, internalisation and ligand binding are expected to all contribute to the observed changes in recruitment of various adaptor proteins to the FGFR2. This supports the idea that

Apert syndrome is not purely a result of increased affinity of the receptor for FGF ligand. Instead the two point mutations in the extracellular domain greatly change numerous receptor properties, which in turn combine to cause significant changes in the recruitment of signalling proteins and subsequently result in the manifestation of Apert syndrome.

5.3.8 Concluding remarks

The data presented in the previous chapter revealed a number of effects of the Apert syndrome mutations on the FGFR2, including its phosphorylation and glycosylation as well as its ligand binding properties. The data presented in this chapter complement these findings and revealed that the changes in the receptor affect not only the activation of one of the major downstream signalling pathways (namely the Erk1/2 pathway), but also result in greatly altered protein recruitment and phosphorylation. This altered recruitment of adaptor proteins such as Shc (shown herein), FRS2 ([224], in submission) and Grb2 (Z. Ahmed, unpublished results) provides a mechanism for the manifestation of Apert syndrome that is different from just 'increased activation' of receptor signalling due to an increased affinity of the receptors for ligand.

Despite it not being clear how exactly such altered protein recruitment is attained, the fact that the Apert syndrome mutations affect the recruitment of signalling proteins to the FGFR2 highlights the importance of correct protein complex assembly to activate the desired signalling pathway (i.e. at the correct magnitude and for the required duration). The importance of this was particularly obvious in the case of PC12 cells, in which the cellular behaviour in response to FGF stimulation was greatly affected by the enhanced Erk1/2 activation in cells expressing the Apert syndrome mutations. As described in Chapter 3, the enhanced activation of the Erk1/2 pathway resulted in proliferation instead of differentiation, which is the normal cellular response to FGF stimulation. Thus even if the Apert mutations initially only affected recruitment of a single protein to the receptor this would have a knock-on effect on recruitment of all proteins normally assembled into an FGFR2-specific complex. Most importantly, the previously described gain-of-function [173] that has been attributed to the Apert mutations seems to be a result of altered protein recruitment and complex formation rather than simply increased signal transduction via the same 'pathways' due to increased affinity of the receptor for ligand.

Chapter 6

Recruitment of Shc to the FGFR2 signalling complex requires the SH2 and PTB domains

6.1 Introduction

Despite various attempts, few proteins that associate directly with the FGFR1 have been identified. Of the six major sites subject to autophosphorylation (refer to Figure 3.9), PLCγ binds to pY766 and Crk binds to Y463, whereas FRS2 binds to the receptor constitutively and independently of tyrosine phosphorylation in the juxtamembrane region. The proteins Sef and Grb14 have also been shown to bind directly to the FGFR1, but their binding sites have not been described [108, 122, 289, 290]. Grb2 is able to bind to the EGFR directly via its SH2 domain, but such an interaction could not be detected in case of the FGFR [104]. FRS2 has been widely accepted as the main docking protein recruiting Grb2 to the FGFR and is subsequently a major component in activation of the Ras-Raf-MEK pathway downstream of the FGFR [9]. However, knockout mice lacking functional FRS2 display no defect in FGFR1 signalling during somitogenesis and gastrulation, which indicates that other proteins such as Shc must be sufficient for activation of the Erk1/2 pathway downstream of the activated FGFR at least under certain circumstances [281].

Numerous studies have shown that Shc is phosphorylated in response to FGF stimulation of cells expressing the FGFR1, FGFR3 or FGFR4 [100, 101, 103, 104, 278, 291]. Co-precipitation of Shc was only demonstrated in the case of the FGFR3 [106] and the FGFR1 in mammalian cells expressing v-Src [105]. The latter, although not a physiologically accurate system, indicated that direct interaction between the receptor and Shc is possible and that a Shc binding site is present on the FGFR1. The Shc SH2 domain has been shown to block Erk2 activation downstream of the *Pleurodeles* FGFR1 (which is homologous to the human FGFR1) in *Xenopus laevis* oocytes, which does not necessarily indicate direct binding, but suggests that such an event occurs via the SH2 domain. A direct interaction between full-length Shc and the FGFR1 has not been described in mammalian cells under more physiological conditions despite reports that various FGFR-derived peptides are able to bind to the Shc SH2 and PTB domains [104, 282, 292].

Despite several reports outlining the involvement of Shc in FGFR signalling, its exact role and perhaps more importantly, the way in which it is recruited to the receptor remains unknown. Shc is able to bind to receptors such as the EGFR, TrkA, insulin receptor or PDGFR via either its SH2 or PTB domain, or a combination of both [50, 71,

293], but direct interaction with the FGFR in vivo has not been shown conclusively. Various studies failed to detect interaction of Shc with the FGFR [103, 104]. A recent report suggested a possible binding site on FGFR1 for the Shc PTB domain. Studies showed that the phospho-peptide based on the sequence surrounding Y730 was able to block its mitogenic function in FGFR1 signalling [282]. Sequence alignment of the FGFR1 and FGFR2 confirmed that Y730 and the surrounding amino acid sequence are conserved between the two receptors. On the other hand, the Shc SH2 domain has been shown to bind both the EGFR and the PDGFR directly [49, 50, 293], and may therefore play a role in Shc recruitment to the FGFR alone or in addition to the PTB domain. Synthetic peptides corresponding to the sequence surrounding phosphorylated Y766, Y730 and Y558 can bind to the Shc SH2 domain [104, 292]. The absence of reports indicating the direct interaction of Shc with the FGFR may be due to the fact that the interaction is of low affinity or only occurs transiently, which makes detection by methods such as co-immunoprecipitation difficult.

In addition to various reports having revealed a role for Shc downstream of the FGFR, the data presented in Chapter 3 confirmed that Shc is involved in FGFR signalling, although its exact role and mode of recruitment to the activated receptor remain elusive. On the basis of the observation that Shc co-localises with the FGFR2, it was chosen to investigate whether these two proteins interact directly with each other and if so, which domain of Shc is critical for this interaction. Co-localisation studies were used to gain insight into which Shc domains were found in the same cellular location as the receptor. Although useful, cellular co-localisation of two proteins does not necessarily indicate direct interaction of the two proteins analysed. Immunoprecipitation and pulldown experiments were also not able to conclusively indicate which Shc domain mediated recruitment to the receptor. Fluorescent lifetime imaging (FLIM) was therefore chosen as a technique that allows investigation of the potential direct interaction between Shc and the FGFR2 intracellularly.

6.2 Results

6.2.1 Co-precipitation of Shc and the FGFR2

Co-localisation of Shc with the wild type FGFR2 was detected in both HEK 293T and PC12 cells (Figure 5.5 and data not shown) and the FGFR2 was observed in Shc immunoprecipitates previously (Figure 5.3). On the basis of these observations, the interaction between Shc and the FGFR2 was investigated in more detail. Shc and the FGFR2 could be co-precipitated. Shc was detected following immunoprecipitation of the FGFR2 using an anti-GFP antibody and, vice versa, the FGFR2 was present in Shc immunoprecipitates (Figure 6.1).

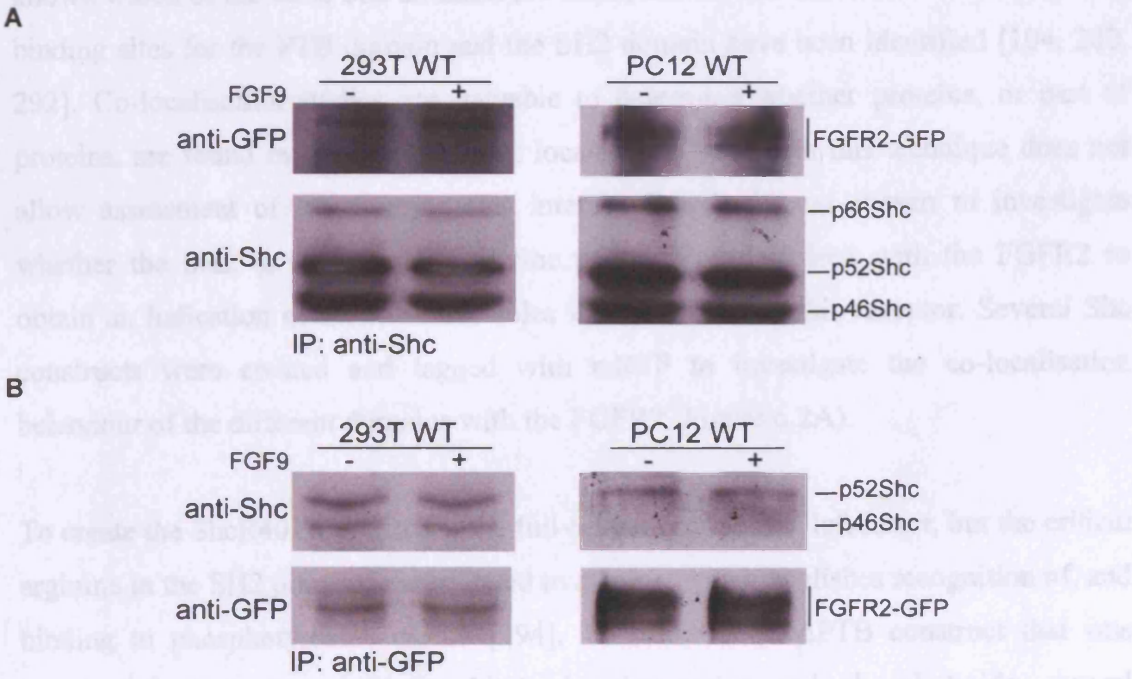


Figure 6.1: Shc and the FGFR2 can be co-immunoprecipitated HEK293T and PC12 cells expressing the wild type FGFR2-GFP were serum-starved and stimulated with 10ng/ml FGF9 for 15 minutes. Cells were lysed and lysates (2mg) were subjected to immunoprecipitation using an anti-Shc antibody (A) or an anti-GFP antibody (B). The western blots were probed with the respective other antibody and then stripped and re-probed with an anti-Shc or anti-GFP antibody to indicate even precipitation of the respective target proteins.

Similar results were obtained for both HEK 293T and PC12 cells, confirming that this observation was not cell-specific. The fact that fairly low amounts of proteins were co-precipitated with the respective binding partner could be due to a number of reasons such as weak affinity or interaction of only a limited pool of receptor with Shc. Such

limited interaction could be a result of low stoichiometry of binding site phosphorylation or blocking of the Shc binding site by other FGFR2-interacting partners. Particularly, a low affinity of this interaction would make detection by immunoprecipitation difficult, because cell lysis disrupts the cellular environment and thereby alters the relative protein concentrations, which may result in dissociation of low affinity protein interactions. Consequently the relatively low levels of protein detected do not rule out direct interaction between these two proteins.

6.2.2 Construction and expression of Shc-RFP constructs

The exact role of Shc in FGFR signalling has not been fully elucidated, and it is not known which of the three Shc domains are important for this function. Several potential binding sites for the PTB domain and the SH2 domain have been identified [104, 282, 292]. Co-localisation studies are valuable to determine whether proteins, or part of proteins, are found in the same cellular localisation. Although this technique does not allow assessment of whether proteins interact directly it was chosen to investigate whether the SH2 or PTB domain of Shc primarily co-localised with the FGFR2 to obtain an indication of their relative roles in recruitment to this receptor. Several Shc constructs were created and tagged with mRFP to investigate the co-localisation behaviour of the different domains with the FGFR2 (Figure 6.2A).

To create the ShcR401A construct, the full-length protein was left intact, but the critical arginine in the SH2 domain was mutated to alanine, which abolishes recognition of, and binding to phosphotyrosine motifs [294]. To create a ShcΔPTB construct that was impaired in both the ability to bind phosphotyrosine and phospholipids, several mutations would have had to be made [185]. Thus instead of completely altering the domain make-up and possibly greatly affecting domain folding and structure, the domain was simply removed from the construct altogether. This seemed to be the most efficient method to completely abolish the ability to bind both phospholipid and phosphotyrosine simultaneously. The 3F construct was created to assess the importance of Shc phosphorylation on co-localisation and interaction with the FGFR2. All three sites known to become tyrosine phosphorylated, Y239/Y240 and Y317, were replaced with phenylalanine. All constructs were transiently transfected into HEK 293T cells and lysates were subjected to SDS-PAGE and immunoblotting with an anti-RFP and an anti-Shc antibody to ensure that the constructs expressed fusion proteins of the correct

molecular weight (Figure 6.2B: top and bottom panel respectively). All constructs led to expression of a protein of the expected molecular weight that could be detected with both antibodies. The PTB domain on its own could not be detected with the anti-Shc antibody, because all anti-Shc antibodies available in the laboratory were raised against the SH2 domain.

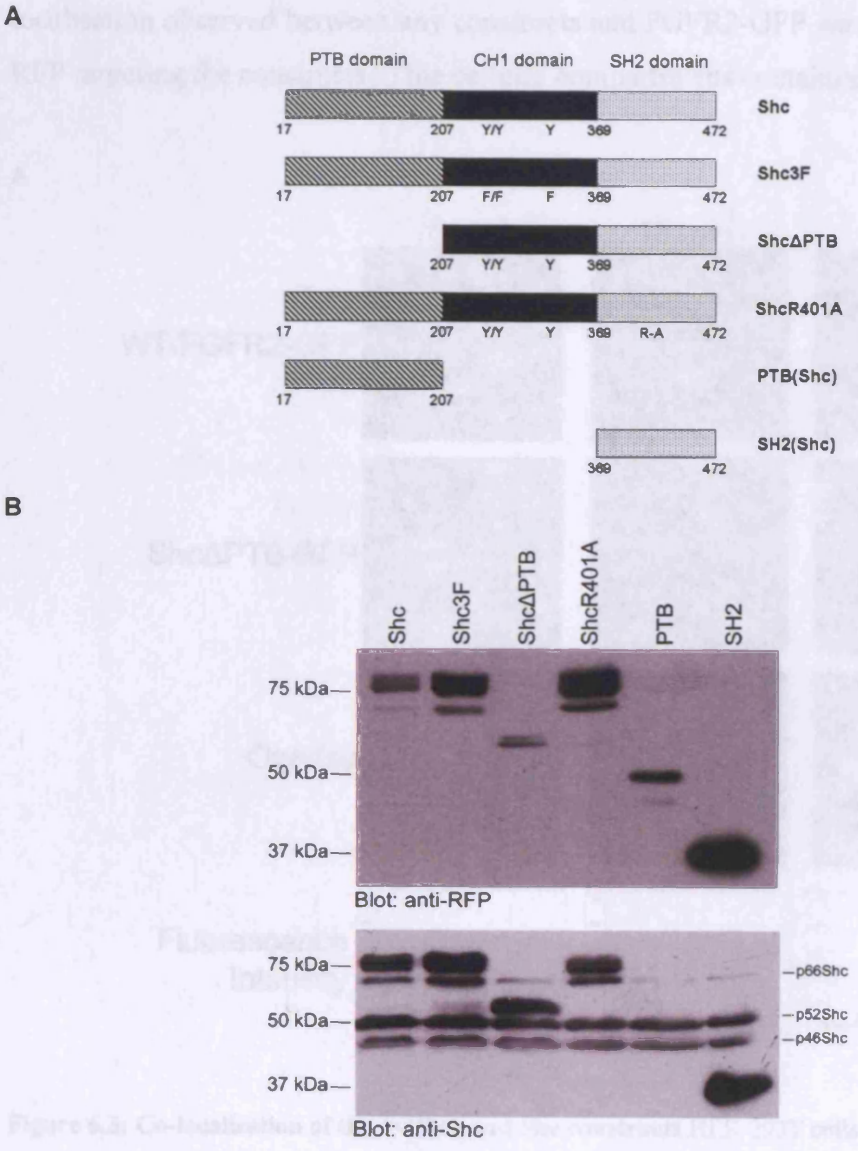


Figure 6.2: Expression of various Shc-RFP constructs The various Shc constructs created are represented in diagrammatic form (A). HEK 293T cells were transiently transfected with the six different RFP-tagged constructs using calcium phosphate precipitation. The cells were lysed and 200μg of the lysates subjected to SDS-PAGE and immunoblotting with an anti-RFP (top panel) and an anti-Shc (bottom panel) antibody (B).

6.2.3 Co-localisation of the FGFR2 with various Shc domains

To determine whether the PTB or SH2 domain of Shc were responsible for observed co-localisation with the FGFR2 (Figure 5.5A), the various Shc constructs were transiently transfected into HEK 293T cells stably expressing the wild type GFP-tagged FGFR2. As a control, cells expressing the wild type FGFR2-GFP were also transfected with RFP on its own, which portrayed no co-localisation with the FGFR2 (Figure 5.5D). Co-localisation observed between any constructs and FGFR2-GFP was therefore not due to RFP targeting the constructs to the cellular compartments containing the FGFR2.

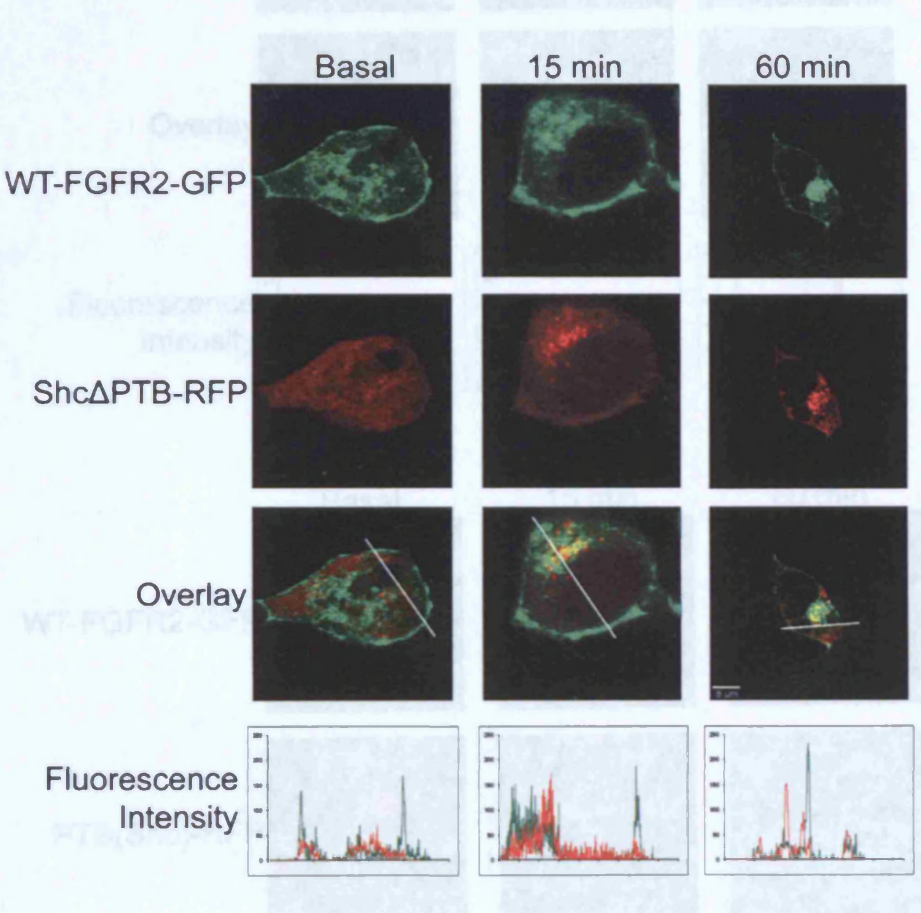
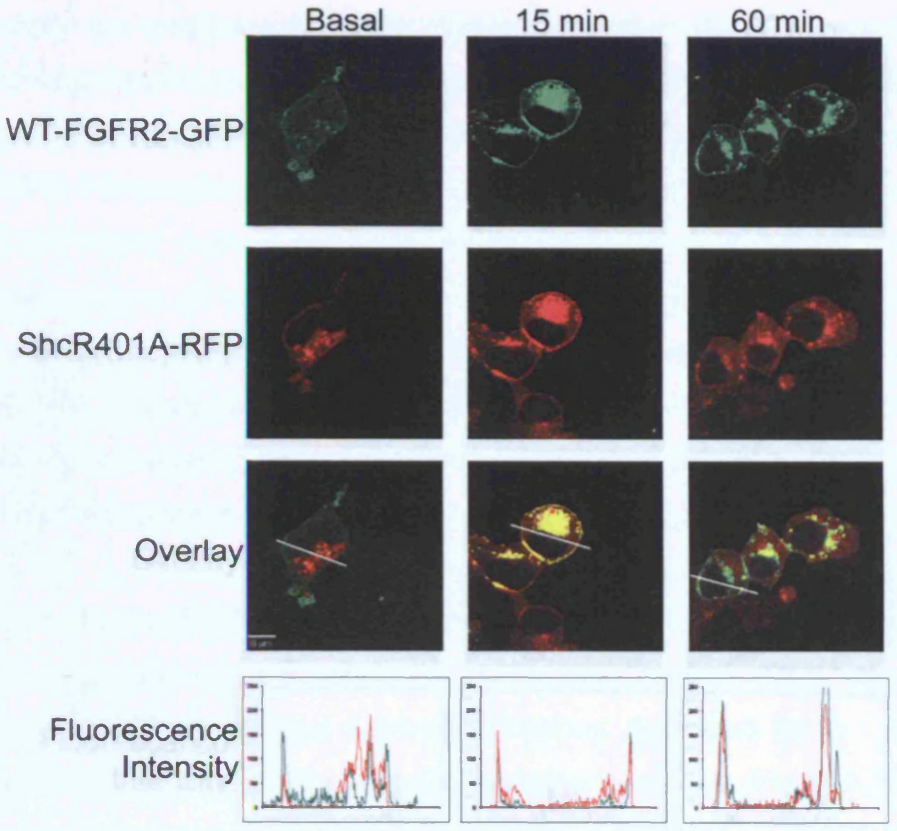


Figure 6.3: Co-localisation of the FGFR2 and Shc constructs HEK 293T cells expressing FGFR2-GFP were transiently transfected with each Shc construct using Lipofectamine 2000. Cells were seeded on glass coverslips, serum-starved overnight, stimulated with FGF9 as indicated and fixed in 4% paraformaldehyde. Confocal microscopy using a Leica SP2 system was carried out. Data shown is representative of at least five cells imaged for each cell line and time point. ShcΔPTB (A), ShcR401A (B, page 191), PTB domain (C, page 191), SH2 domain (D, page 192), Shc3F (E, page 192). The relative fluorescence intensities of GFP and RFP along an arbitrarily drawn line are represented graphically in green and red respectively.

B


Basal
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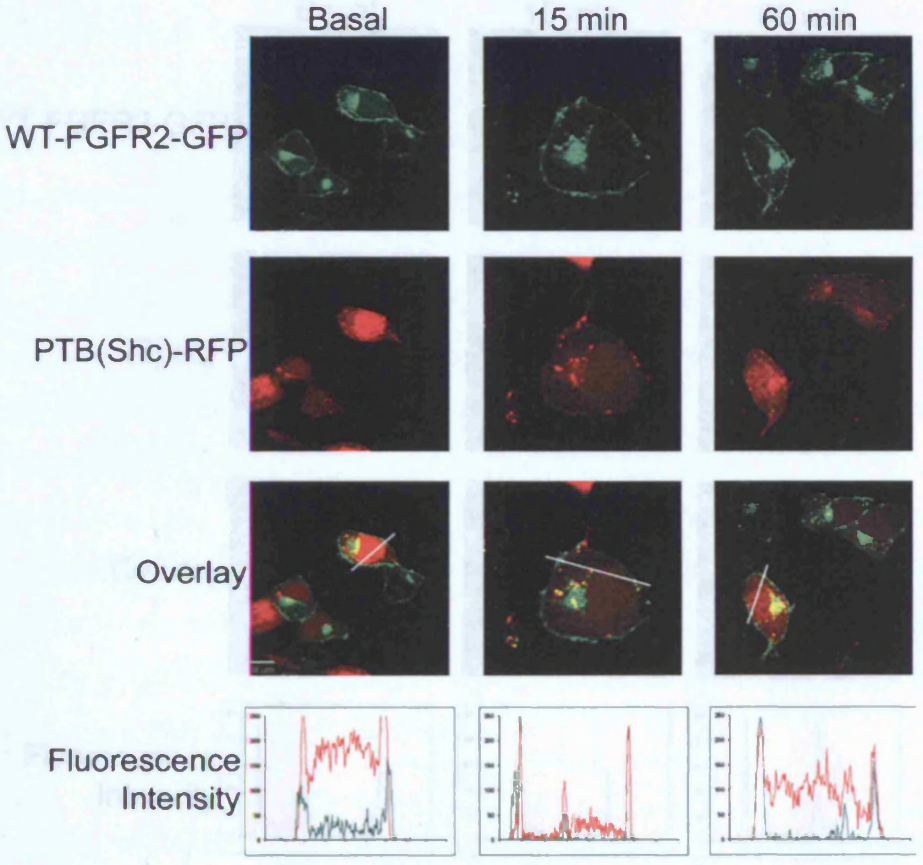
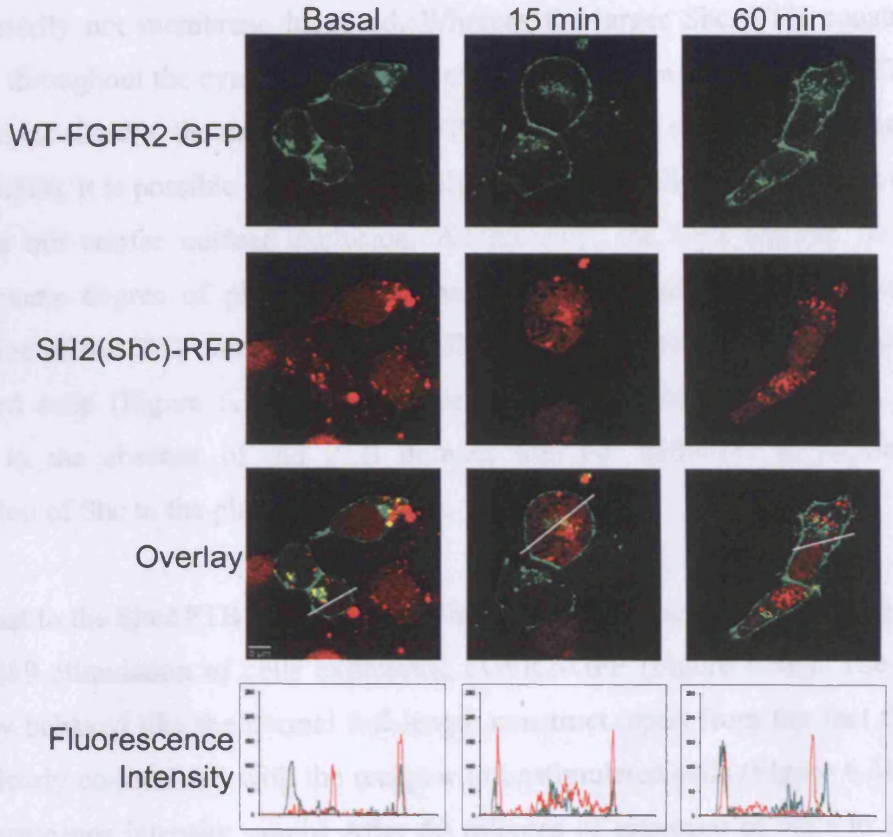
60 min
WT-FGFR2-GFP
ShcR401A-RFP
Overlay
Fluorescence Intensity

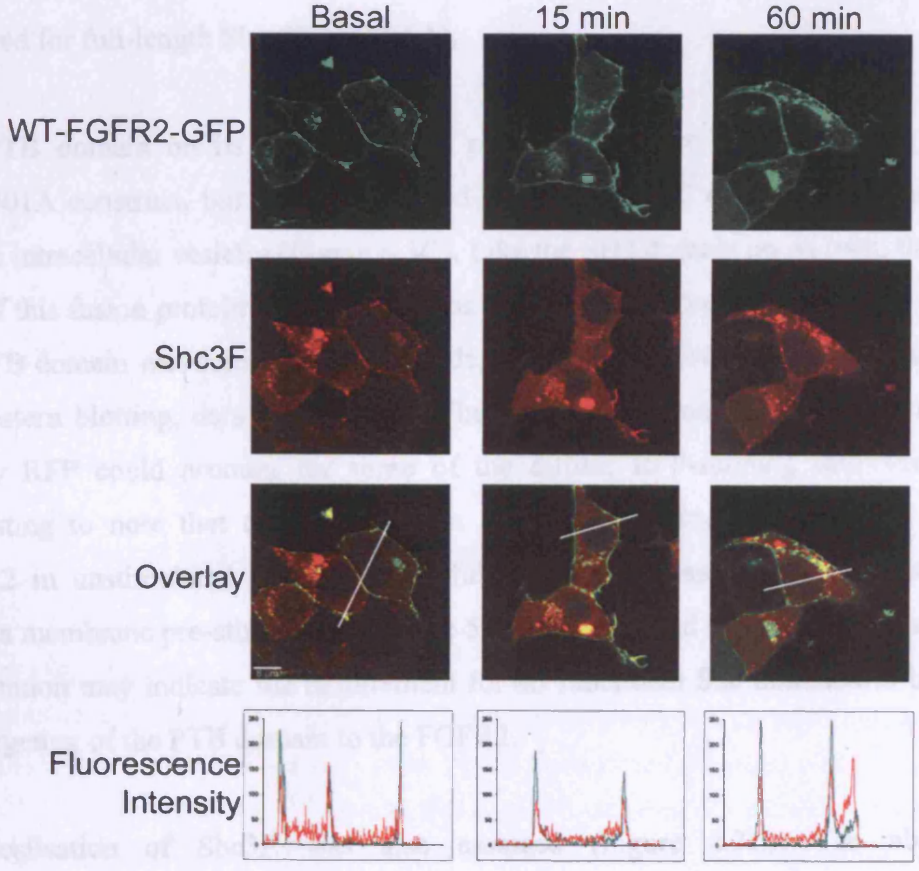
C


Basal
15 min
60 min
WT-FGFR2-GFP
PTB(Shc)-RFP
Overlay
Fluorescence Intensity

D


Basal
15 min
60 min
WT-FGFR2-GFP
SH2(Shc)-RFP
Overlay
Fluorescence Intensity

E


Basal
15 min
60 min
WT-FGFR2-GFP
Shc3F
Overlay
Fluorescence Intensity

The SH2 domain and the ShcΔPTB construct (Figure 6.3D and A respectively) were both primarily not membrane localised. Whereas the larger ShcΔPTB construct was localised throughout the cytoplasm but was excluded from the nucleus, the SH2 domain alone also localised to the nucleus. Since RFP on its own was also located in the nucleus (Figure 5.5D), it is possible that this is simply due to the smaller nature of this construct that does not confer nuclear exclusion. Additionally, the SH2 domain on its own showed some degree of plasma membrane localisation and a small amount of co-localisation of the SH2 domain with the FGFR2 could be detected in unstimulated and stimulated cells (Figure 6.3D: fluorescence intensity graphs). Nonetheless, the SH2 domain in the absence of the PTB domain was not sufficient to mediate major localisation of Shc to the plasma membrane.

In contrast to the ShcΔPTB construct, the ShcR401A construct was membrane localised after FGF9 stimulation of cells expressing FGFR2-GFP (Figure 6.3B). The construct generally behaved like the normal full-length construct, apart from the fact that it was not as clearly co-localised with the receptor in unstimulated cells (Figure 6.3B: panel g and fluorescence intensity graph) After 60 minutes of exposure of cells to FGF9, the two proteins still co-localised, albeit more on intracellular vesicles, as was previously observed for full-length Shc (Figure 5.5A).

The PTB domain on its own generally portrayed a more diffuse pattern than the ShcR401A construct, but also co-localised with the FGFR2 on the plasma membrane and on intracellular vesicles (Figure 6.3C). Like the SH2 domain on its own, the smaller size of this fusion protein meant that it was not excluded from the nucleus. In addition, the PTB domain was somewhat prone to degradation when over-expressed (as assessed by western blotting, data not shown). The presence of smaller fragments containing mostly RFP could account for some of the diffuse RFP-staining observed. It was interesting to note that the PTB domain also did not strongly co-localise with the FGFR2 in unstimulated cells, whereas full-length Shc was already localised at the plasma membrane pre-stimulation (Figure 5.5A: panels f and k). This lack of strong co-localisation may indicate the requirement for all functional Shc domains to be present for targeting of the PTB domain to the FGFR2.

Co-localisation of Shc3F was also assessed (Figure 6.3E). The absence of phosphorylation did not prevent Shc from co-localising with the FGFR2. Overall, the

co-localisation pattern was very similar to that of full-length Shc: membrane localisation was somewhat decreased after 15 minutes of FGF9 stimulation but the FGFR2 and Shc3F greatly co-localised 60 minutes post-stimulation (Figure 6.3E: panels g and i respectively). These findings indicate that Shc recruitment to the FGFR2 is not an event that occurs as a result of phosphorylation. More likely the reverse occurs and membrane targeting is required for phosphorylation by the receptor to occur.

6.2.4 Co-precipitation analysis of the interaction of individual Shc domains with the FGFR2

Even though co-localisation studies provide a good indication of whether two proteins are found in the same region of a cell, the resolution achieved by confocal microscopy is not high enough to allow observations regarding direct proteins-protein interactions to be made. To confirm whether the different constructs and the receptor demonstrated the same co-precipitation as co-localisation patterns, cells expressing the wild type FGFR2 were transiently transfected with the various Shc constructs and cell lysates of stimulated and unstimulated cells were subjected to anti-GFP immunoprecipitation.

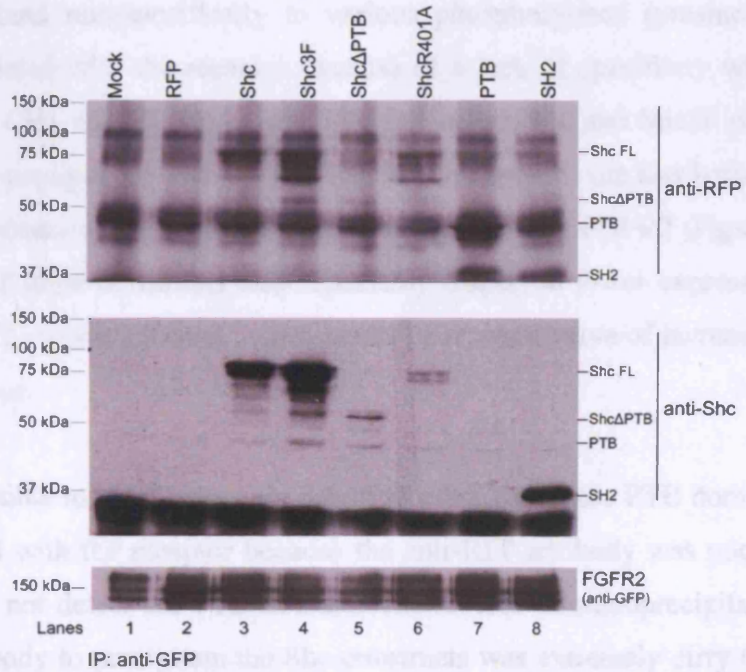


Figure 6.4: Co-precipitation of FGFR2 and Shc constructs HEK 293T cells expressing FGFR2-GFP were transiently transfected with equal amounts of each Shc-construct as well as RFP on its own using calcium phosphate precipitation. Cells were serum-starved overnight and stimulated with 10ng/ml FGF9 for 15 minutes. Cell lysates were immunoprecipitated with an anti-GFP antibody overnight and precipitants were subjected to SDS-PAGE and immunoblotting using anti-RFP, anti-Shc and anti-GFP antibodies. Shc FL: Shc full length

Following immunoprecipitation, the western blots were probed with an anti-RFP antibody (Figure 6.4: upper panel). Unfortunately, the antibody detected a large number of non-specific background bands of similar size as some of the constructs. This made identification of whether or not a certain RFP-tagged construct was co-precipitated with the GFP-tagged receptor very difficult and inaccurate. Consequently, the same blot was re-probed using an anti-Shc antibody, which excluded many of the background bands (Figure 6.4: middle panel). All constructs co-precipitated with the receptor, which was in contrast with the results obtained from the co-localisation studies. However, the PTB domain and ShcR401A only precipitated to a very small extent compared to their expression levels (Figure 6.2), whereas full-length Shc, Shc3F and the SH2 domain co-precipitated with the receptor very well. It is possible that the SH2 domain is normally excluded from interaction with the receptor in vivo, because the binding of specific binding partners is highly regulated in the cellular context. In the cell lysate changes in the relative protein concentration might occur which might cause interactions to become somewhat more dynamic. As a result the SH2 domain on its own or the SH2 domain of the ShcΔPTB construct could co-precipitate with the receptor, despite minimal co-localisation having been observed (Figure 6.3D). Particularly the individual SH2 domain may bind non-specifically to various phosphorylated tyrosines on different proteins associated with the receptor because of a lack of specificity when not in the context of the CH1 and PTB domains. The full-length Shc and Shc3F constructs most dominantly co-precipitated with the FGFR2, which may indicate that both the functional SH2 and PTB domains are required for interaction with the FGFR2 (Figure 6.4: middle panel). Both of these constructs also repeatedly displayed lower expression levels but greatest FGFR2 co-precipitation, which could be representative of increased association with the receptor.

It was not possible to unambiguously detect whether or not the PTB domain on its own co-precipitated with the receptor because the anti-RFP antibody was poor and the Shc antibody does not detect the PTB domain. The reverse immunoprecipitation using the anti-RFP antibody to precipitate the Shc constructs was extremely dirty (due to a large amount of background immuno-reactivity of the antibody) and unambiguous analysis of whether or not the FGFR2 was co-precipitated was not possible.

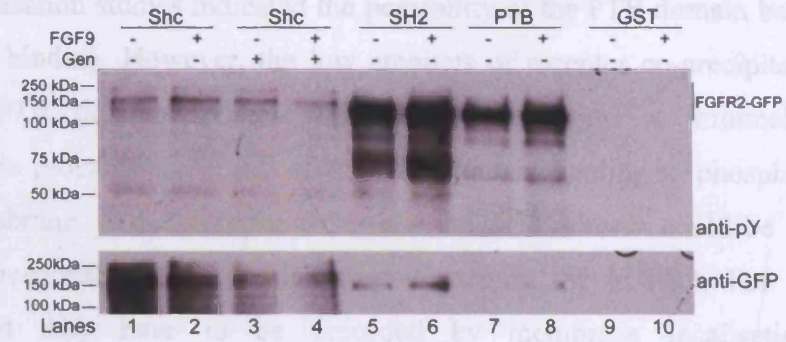


Figure 6.5: Pulldown of the FGFR2 using GST-tagged Shc and Shc SH2 and PTB domains Full-length Shc-GST, SH2(Shc)-GST and PTB(Shc)-GST on glutathione sepharose were incubated with HEK 293T WT cell lysates from unstimulated and FGF9-stimulated cells overnight. The kinase inhibitor genistein was added to full length Shc to avoid phosphorylation of SHc by potentially active kinases in the cell lysates. The beads were washed extensively and the precipitated proteins were subjected to SDS-PAGE and immunoblotting with an anti-phosphotyrosine antibody and reprobed with an anti-GFP antibody after stripping the membrane. The same trend was observed in two independent experiments.

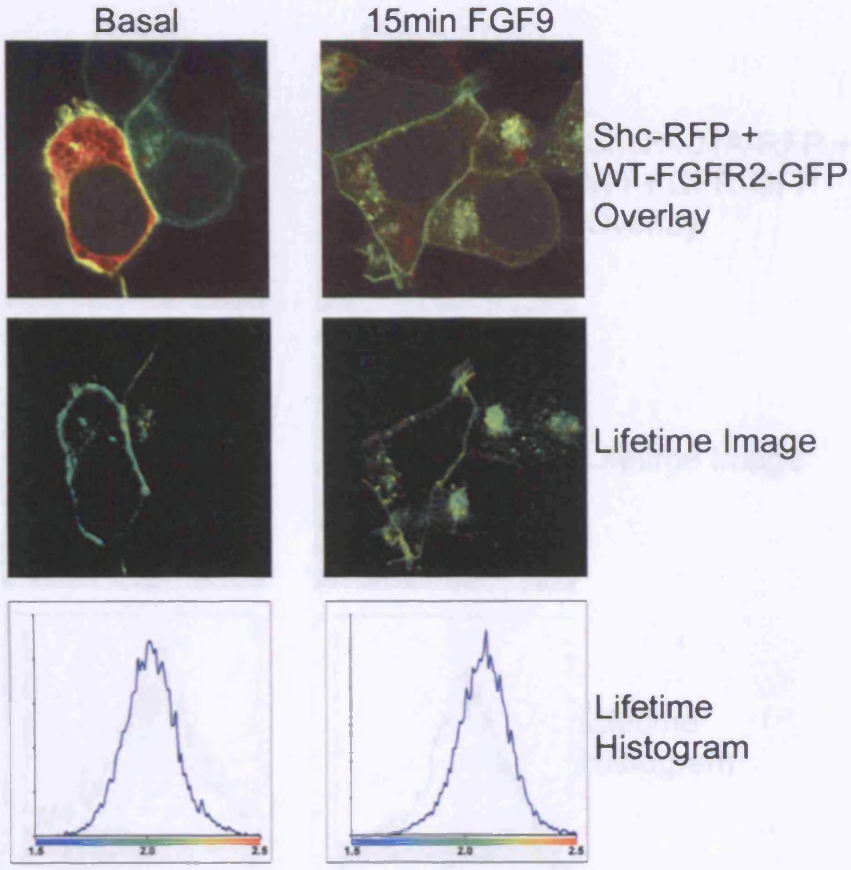
To confirm whether the SH2 domain mediated stronger interaction with the FGFR2 than the PTB domain, GST-tagged full-length Shc and its SH2 and PTB domains were expressed in *E.coli* and used to pull down the receptor from unstimulated and FGF9 stimulated HEK 293T cells expressing the FGFR2-GFP (Figure 6.5). All three constructs were able to co-precipitate the FGFR2. However, the SH2 domain was able to precipitate the FGFR2 much more efficiently than the PTB domain (Figure 6.5: anti-GFP blot lower panel), which is in agreement with the RFP-immunoprecipitation experiment (Figure 6.4). The PTB domain was able to interact with the receptor, but only to a minimal extent. Precipitation of the receptor by full-length Shc was more efficient than by either domain alone, despite the fact that a lot less GST-fusion protein had to be used since bacterial expression of full-length Shc was very poor and low yields of recombinant protein were obtained. The observation that neither full-length Shc nor any of the individual domains could interact with the fully glycosylated receptor, indicates the presence of some mechanism regulating interaction of Shc with the FGFR2. For correct and efficient interaction all Shc domains are required to be present. The kinase inhibitor genistein was added to full-length Shc to avoid possible phosphorylation by active kinases in the lysates. This did not have any effect on FGFR2 co-precipitation (Figure 6.5: lanes 3-4 versus 1-2), which suggests that phosphorylation has no effect on interaction with the receptor. This confirms the results obtained from the RFP-immunoprecipitation and co-localisation experiments (Figures 6.3E and 6.4).

The co-localisation studies indicated the possibility of the PTB domain being important for receptor binding. However, the low amounts of receptor co-precipitation with the individual PTB domain indicate that direct interaction is minimal. Membrane localisation is probably the result of the PTB domain binding to phospholipids in the plasma membrane. If this was the case then the SH2 domain could be the main Shc component responsible for complex formation with the FGFR2. For this to occur efficiently it may have to be preceded by membrane localisation via PTB domain/phospholipids interaction, which could explain the respective absence or low level of co-localisation of the ShcΔPTB and SH2 domain with the receptor. The SH2 domain might also require presence of the PTB domain for efficient interaction with the receptor, since full-length GST- and RFP-Shc were more strongly associated with the receptor than individual domains or incomplete protein constructs (Figures 6.4 and 6.5).

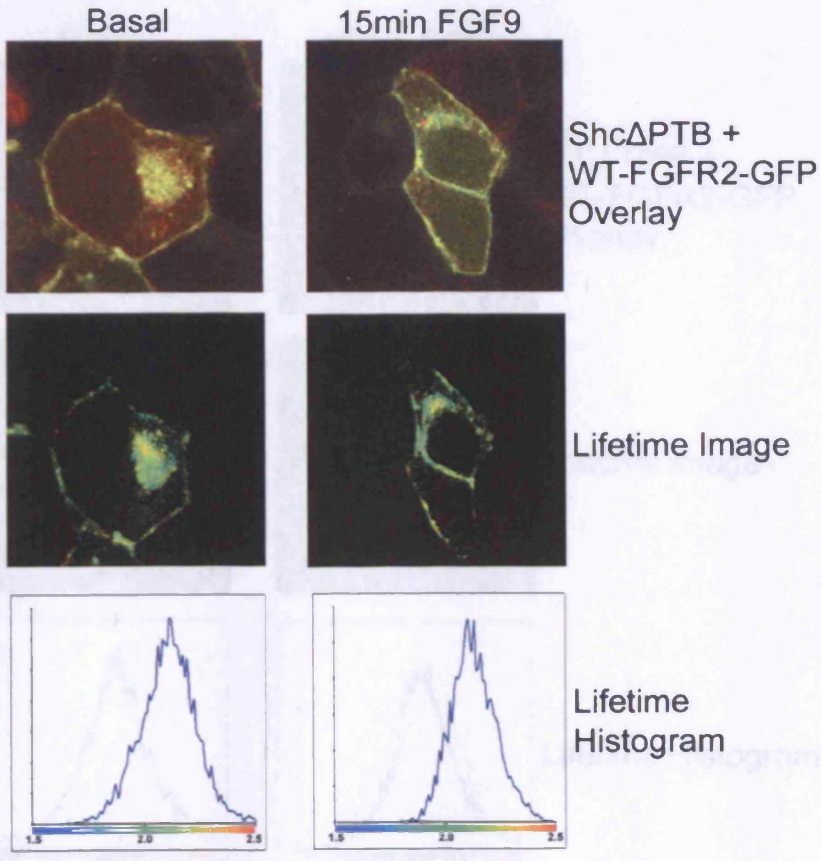
6.2.5 FLIM analysis revealed the absence of direct interaction between Shc and the FGFR2

To resolve discrepancies arisen by the use of different techniques and to confirm the hypothesis that the PTB domain is required for membrane localisation but the SH2 domain interacts with the receptor, fluorescence lifetime imaging microscopy (FLIM) was employed. GFP on its own portrayed an average lifetime of around 2.1ns, which did not alter significantly in the presence of RFP (Figure 6.6G: left and middle panels respectively). The lifetime histogram represents the lifetime of all pixels within the image taken, and thereby indicates the distribution across the whole cell. The overall lifetime of GFP was fairly uniform, tightly centred around the peak at 2.1ns. The FGFR2-GFP shows a slightly different pattern on the lifetime histogram (Figure 6.6G: right panel). Although still centred around a peak of 2.1, the shape of the curve is much broader with various smaller peaks present. These changes may be due to differences in the local environment throughout the cell, which may affect GFP lifetime locally [295]. Differences in membrane or juxtamembrane environment might affect the lifetime of GFP and therefore create regions in which lifetimes shorter or longer than 2.1ns were detected.

A


Basal
15min FGF9
Shc-RFP +
WT-FGFR2-GFP
Overlay
Lifetime Image
Lifetime
Histogram
1.5
2.0
2.5

B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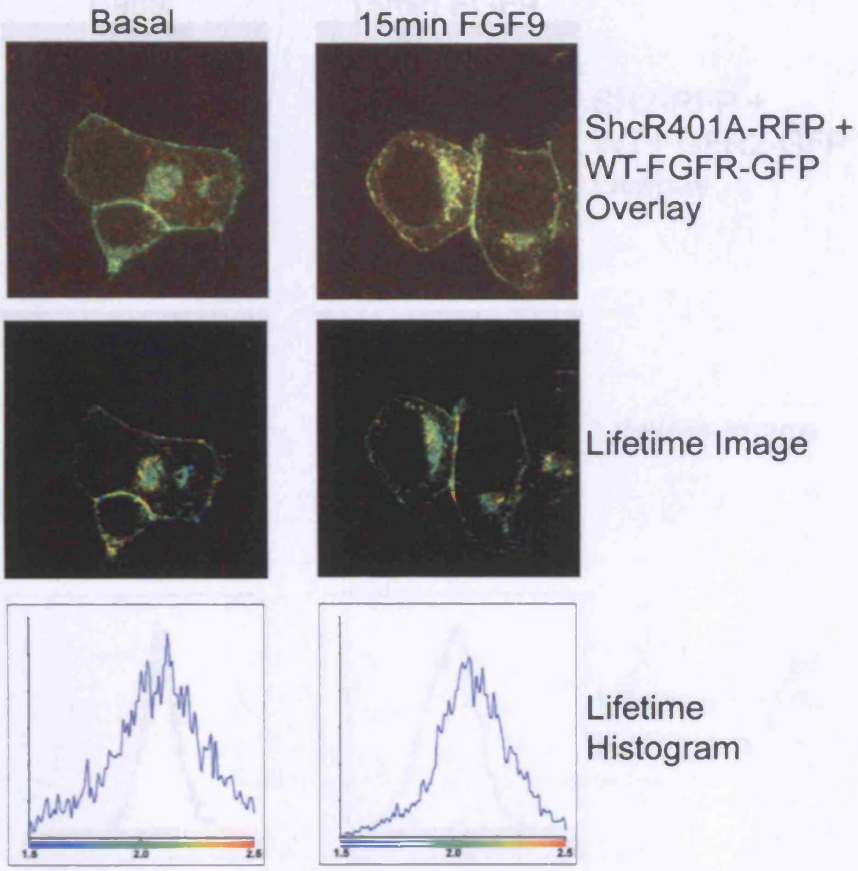
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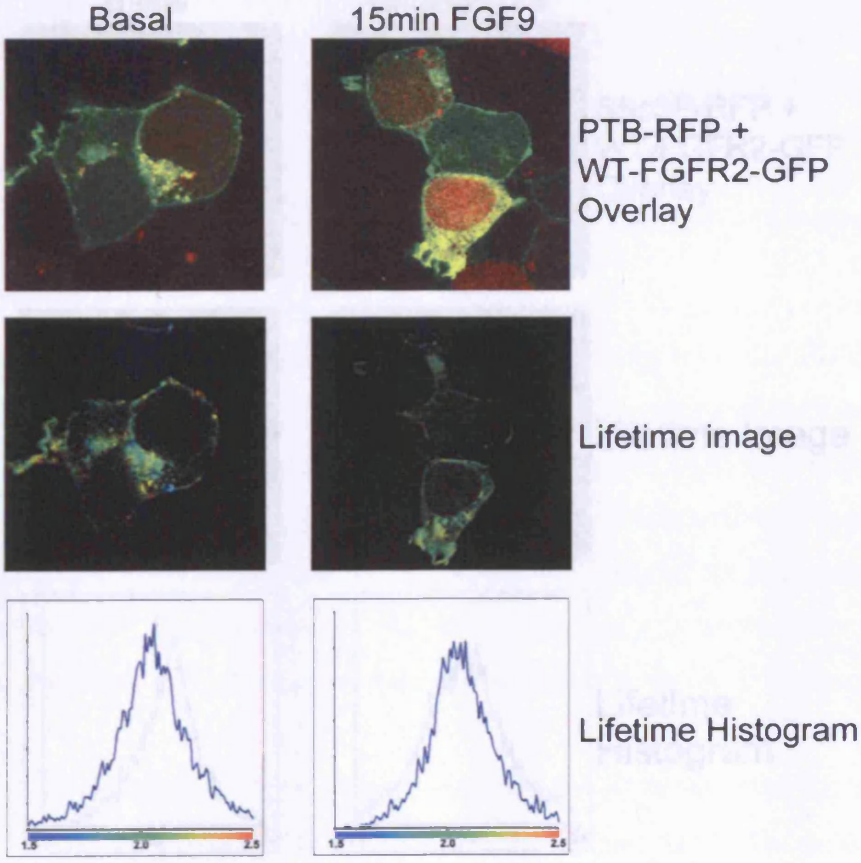
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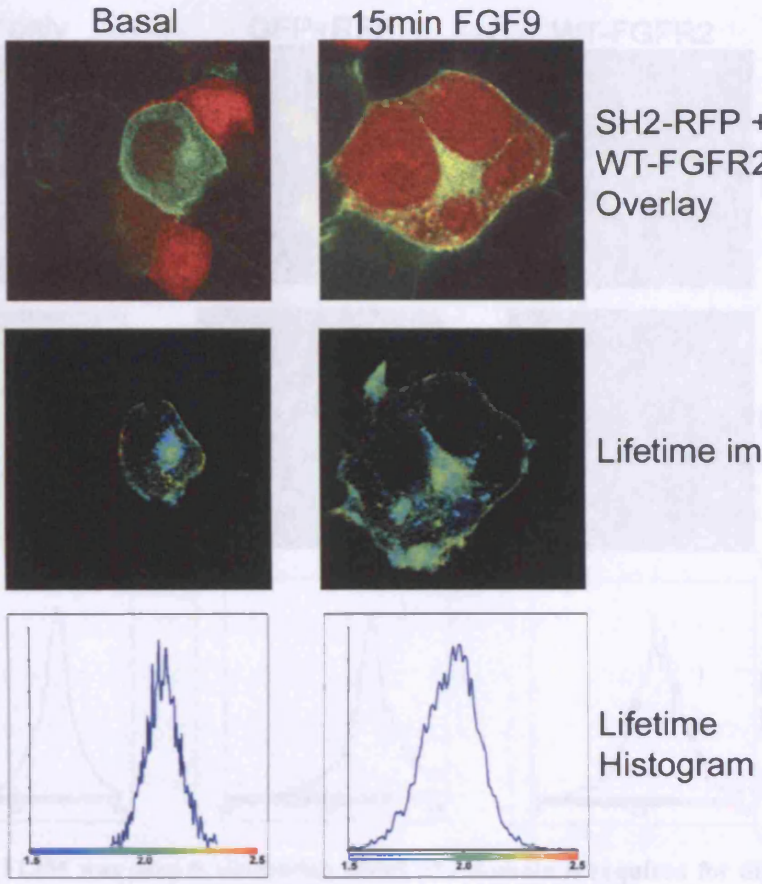
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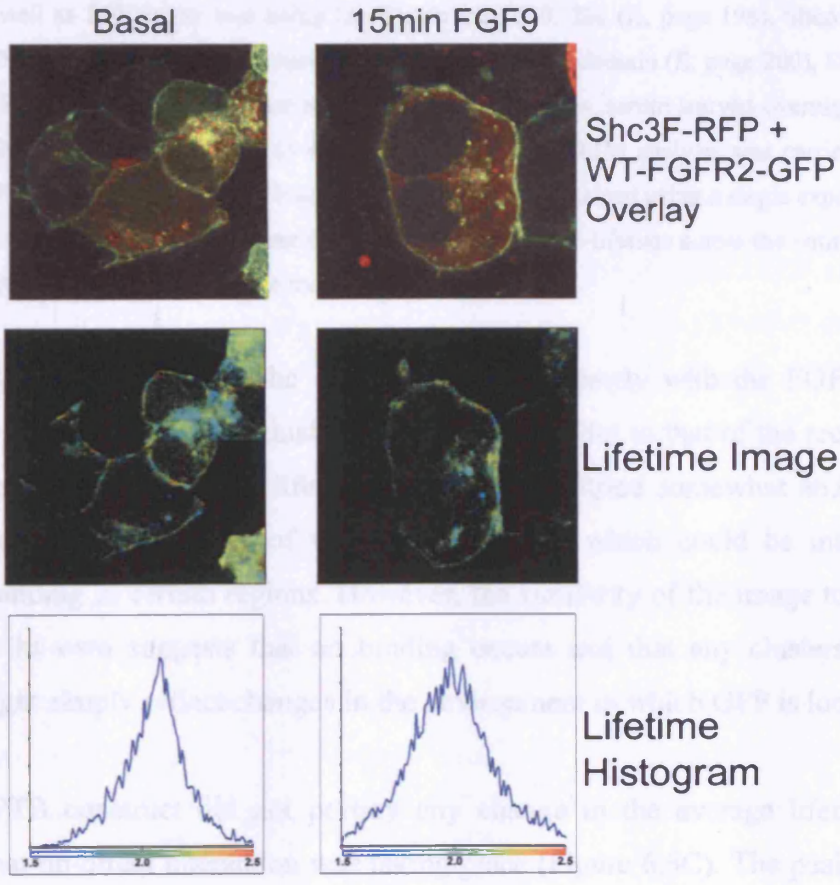
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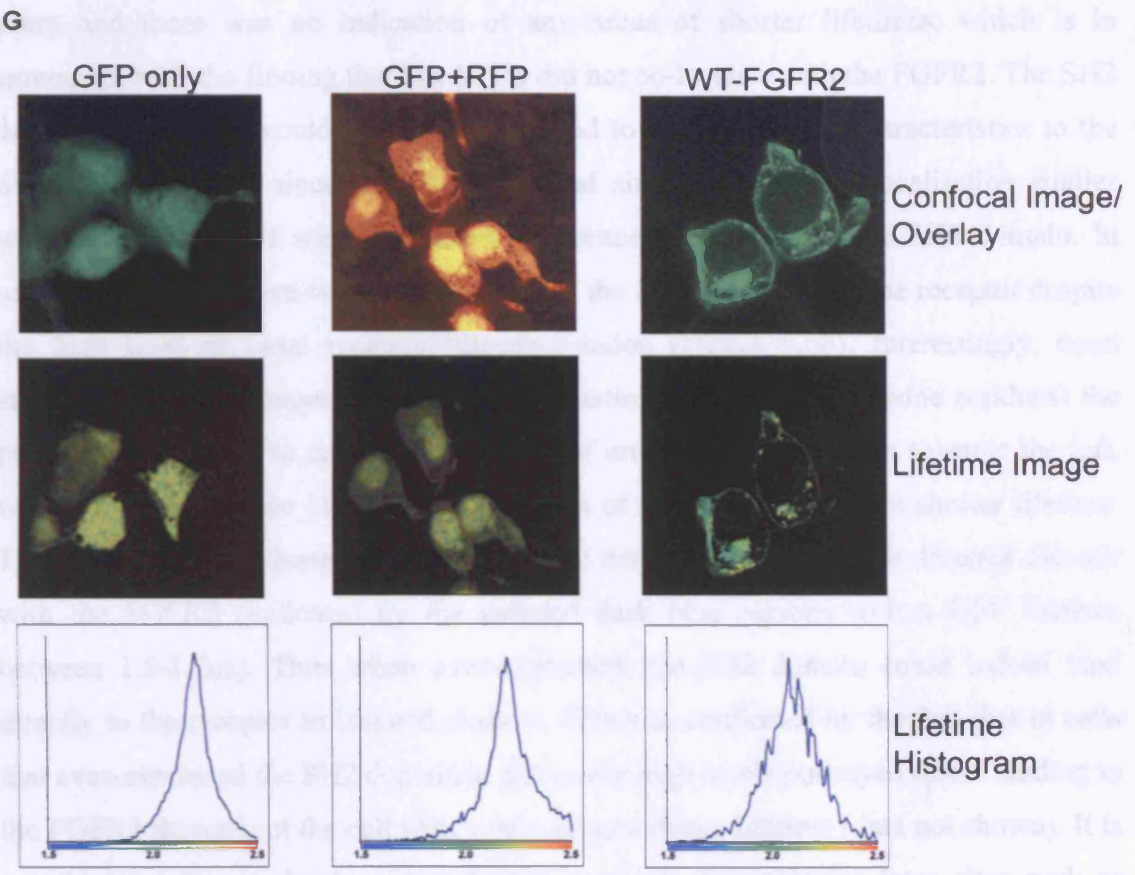

Figure 6.6: FLIM was used to determine which Shc domain is required for direct interaction with the FGFR2 HEK 293T cells expressing FGFR2-GFP were transiently transfected with each Shc-construct as well as RFP on its own using Lipofectamine 2000. Shc (A, page 198), ShcΔPTB (B, page 198), ShcR401A (C, page 199), PTB domain (D, page 199), SH2 domain (E, page 200), Shc3F (F, page 200), GFP/RFP controls (G). Cells were seeded on glass coverslips, serum-starved overnight, stimulated with FGF9 for 15 minutes and fixed in 4% paraformaldehyde. FLIM analysis was carried out using a Leica TCS SP2 inverted microscope. Fluorescence decays were obtained using a single exponential decay model. The lifetime histograms represent the overall spread of GFP-lifetime across the entire cell. Images shown are representative of at least five independent cells analysed.

FLIM analysis revealed that Shc does not interact directly with the FGFR2 (Figure 6.6A). The peak of the lifetime histogram was very similar to that of the receptor on its own. In unstimulated cells the lifetime average was shifted somewhat and stimulated cells portrayed small pockets of very short lifetime, which could be interpreted as restricted binding in certain regions. However, the similarity of the image to that of the FGFR2 on its own suggests that no binding occurs and that any clusters of shorter lifetime might simply reflect changes in the environment in which GFP is located.

The ShcΔPTB construct did not portray any change in the average lifetime, which indicates that no direct interaction was taking place (Figure 6.6C). The peak was fairly

sharp and there was no indication of any areas of shorter lifetimes, which is in agreement with the finding that ShcΔPTB did not co-localise with the FGFR2. The SH2 domain on its own would have been expected to portray similar characteristics to the ShcΔPTB construct, since these two behaved similarly in the co-localisation studies with the exception of some low level membrane localization of the SH2 domain. In unstimulated cells there was no interaction of the SH2 domain with the receptor despite the high level of basal receptor phosphorylation (Figure 6.6F). Interestingly, upon stimulation (and subsequently the phosphorylation of additional tyrosine residues) the peak shifted slightly to an average lifetime of around 2.0 with a bias towards the left, which is representative of a larger population of molecules with even shorter lifetime. There were isolated clusters in which the SH2 domain was now able to interact directly with the FGFR2 (indicated by the isolated dark blue regions with a GFP lifetime between 1.5-1.7ns). Thus when over-expressed, the SH2 domain could indeed bind directly to the receptor in isolated clusters. This was confirmed by the fact that in cells that over-expressed the SH2 domain at extremely high levels portrayed direct binding to the FGFR2 throughout the cell with a very short average lifetime (data not shown). It is possible that this is due to either displacement of other proteins from sites such as pY766 or due to binding to one of the various tyrosine residues shown to be phosphorylated but lacking an identified binding partner.

It was interesting to note that the ShcR401A and the PTB domains both portrayed isolated clusters of shorter lifetime throughout the cells (Figure 6.6D and E respectively: dark blue spots). Thus although the average lifetime of GFP was only slightly left-shifted compared to that obtained for cells only expressing FGFR2-GFP and no RFP construct, the spread was much larger and a proportion of the overall lifetimes measured in the cell were very short (1.5-1.7ns, particularly in unstimulated cells expressing ShcR401A). It is unclear why the lifetimes are shorter for the ShcR401A construct than they are for the PTB domain on its own. This may simply be due to slight cell-cell variations. Another explanation could be that in isolated regions the binding of the construct to the membrane via the PTB domain positions the RFP molecule in close enough proximity to the GFP moiety attached to the receptor to cause FRET to occur. However, the overall patterns observed were not greatly different from full-length Shc or cells not expressing any RFP-tagged construct. This indicates that no direct interaction of Shc with the FGFR2 via the PTB domain occurs, which correlates with

the fairly low levels of precipitation of the receptor by the GST-PTB domain (Figure 6.5).

The triple tyrosine mutant, Shc3F, showed a similar FLIM pattern to the ShcR401A construct. This was in agreement with the co-localisation patterns observed. However, some more clusters in which shorter GFP lifetime was observed were present compared to the full-length Shc in stimulated cells. It is unclear why this occurs and whether these observations are simply due to cell-cell variation or alterations in the environment surrounding the GFP molecule.

Thus altogether, Shc does not interact with the FGFR2 in vivo. Association of Shc with the FGFR2 therefore has to occur indirectly via interaction with other proteins in larger multiprotein complexes. Overall, the PTB domain, even when over-expressed in isolation, was unable to bind to the receptor. On the other hand, the SH2 domain was able to bind to the receptor when expressed in isolation, although this interaction was prevented in the context of the full-length protein.

6.3 Discussion

6.3.1 The Shc SH2 domain can potentially bind to the FGFR2

FGF9 stimulation of PC12 and HEK 293T cells expressing the FGFR2 was able to induce Shc phosphorylation. Moreover it was found to co-precipitate and co-localise with the receptor in both cell systems (Figures 5.5A, 6.1 and data not shown), indicating a role downstream of the FGFR2. However, FLIM analysis of full-length Shc and various truncated constructs/domains revealed that no direct protein-protein interaction takes place between the FGFR2 and Shc, although the SH2 domain theoretically possesses the ability to interact directly with the receptor when over-expressed in vivo (Figure 6.2E). Klint *et al.* have previously shown that the isolated Shc SH2 domain is able to bind to various FGFR1-derived synthetic peptides, particularly that corresponding to the sequence surrounding Y766 [104]. Another study also implemented the corresponding residue in the chicken bFGFR as the highest affinity binding site for the Shc SH2 domain and proposed that Y728 and Y556 (corresponding to Y730 and Y558 in human FGFR1) were minor Shc SH2 binding sites [292]. Although in vitro studies using isolated bacterially expressed domains and synthetic peptides do not necessarily represent true cellular events, these data provide support for the fact that the isolated SH2 domain may be able to displace PLCγ from its binding site (pY766) or is able to bind to another site when expressed in isolation and present at high concentrations. This would explain the observed interaction between the SH2 domain and the receptor and the high levels of receptor co-precipitation of these two proteins. This interaction must be prevented in the context of the full-length Shc protein since no association of the full-length Shc protein with the FGFR2 was observed (Figure 6.6A). This further indicates that the interaction of the SH2 domain with the receptor is an artefact produced by the overexpression of this single domain in the absence of other, regulatory components of the whole protein.

6.3.2 Full-length Shc does not bind directly to the FGFR2

The Shc PTB domain and a full-length construct with a non-functional SH2 domain both co-localised with the receptor at the plasma membrane (whereas neither the SH2 domain nor the ShcΔPTB construct were able to do so). This indicated an important role for the PTB domain in terms of Shc recruitment to the membrane and the activated receptor. Since the FRS2 PTB domain binding site does not contain the consensus

NPXpY sequence required for Shc PTB domain binding [108] and no such sequence is found on FGFR2, the motif around pY730 could have been a candidate region for Shc PTB domain binding. This region portrays similarities to sequences of known Shc PTB interacting regions and was able to bind Shc when phosphorylated [282]. In this report Dunican *et al.* implicate the PTB domain as the moiety interacting with the pY730 peptide. However, this is not shown conclusively and other studies suggested that this residue could be part of a Shc SH2 domain binding site [104], which could indicate that the binding described by Dunican *et al.* occurs via the SH2 rather than the PTB domain. The FLIM data presented in this study revealed that neither the PTB domain alone nor any other Shc constructs were able to interact with the receptor. Support for the lack of Shc association with the receptor is obtained from the fact that Shc phosphorylation was found to be unaltered in response to stimulation of receptors lacking all major phosphorylation sites [103], which indicates that there is no requirement for FGFR-Shc interaction. The FLIM data have revealed that in the case of the FGFR2, neither Y730 nor any other tyrosine residues form a binding site for Shc in vivo.

6.3.3 Regulation of the interaction of Shc with the FGFR2

Several factors could play a role in preventing the interaction of Shc and the FGFR2 despite the existence of possible binding sites. Firstly, the tyrosine residues that form the basis for proposed/hypothetical Shc SH2 or PTB domain binding sites may not be phosphorylated at all or only to a low extent in vivo. For example, Y730 is stoichiometrically less phosphorylated than other tyrosine residues in the C-terminal tail of FGFR1 [103], and a recent study was unable to detect Y730 phosphorylation at all [15]. The low levels of phosphorylation and the fact that this site is effectively buried in the three dimensional structure of the receptor [296] would make this site unsuitable for Shc PTB or SH2 domain binding. Furthermore, the binding of other proteins such as PLCγ or Crk to the receptor at certain sites may prevent access to potential binding sites even if they were phosphorylated. The clusters in which interaction between the FGFR2 and the SH2 domain were observed using FLIM could be explained by the displacement of PLCγ from the Y766 site or non-specific binding to other phosphorylated tyrosine residues on the receptor. Although the isolated domain may be able to bind different regions on the receptor, this binding is prevented in the context of the whole protein. The reason for this is unclear, but the FGFR system seems to utilise FRS2 as the main Grb2-recruiting protein so that association with Shc is not required. Shc may play

another role downstream of the FGFR2, for which interaction with the receptor needs to prevented so that its SH2 and/or PTB domains are available to interact with other proteins to initiate correct signalling complex formation.

6.3.4 A model for the role of the three Shc domains in recruitment to and indirect interaction with the FGFR2

6.3.4.1 The PTB domain is required for membrane recruitment whereas the SH2 domain mediates interaction with proteins in a signalling complex

Overall, the data presented in this work implement the PTB domain with a role in membrane localisation via binding of phospholipids. Although the PTB domain co-precipitated with the receptor, this interaction seemed to be weak and the PTB domain was unable to interact directly with the receptor. This observation suggests that phospholipid binding is more important in terms of membrane localisation. Previous studies have shown the lack of localisation of the ShcΔPTB construct to the membranous fraction, whereas full-length Shc was associated with the membrane fractions of unstimulated T cells, BaF cells and COS cells [185]. This process was thus mediated by the PTB domain, which is in agreement with the results presented herein (Figure 6.3).

Both phospholipid and phosphotyrosine binding have been shown to be important for Shc phosphorylation in response to IL3 stimulation [185]. Since the Shc PTB domain was also able to bind the FGFR2 (Figure 6.4), albeit not very efficiently or with low affinity, a similar mechanism could take place in FGFR2 signalling. The phosphotyrosine binding event may be secondary to membrane recruitment via phospholipid binding and may be important for stabilisation of the protein in the vicinity of the FGFR2 so that efficient phosphorylation of Shc can occur. Phospholipid binding would therefore allow Shc to be more readily available for recruitment to the receptor. Further studies using full-length Shc with PTB domain mutations abolishing phospholipid or phosphotyrosine binding independently would be able to reveal the importance of each individual property in Shc membrane recruitment and phosphorylation by the FGFR2.

The recruitment of Shc to the plasma membrane via the PTB domain would allow the SH2 domain to be brought to the vicinity of its binding partners in the FGFR2 signalling

complex, followed by their interaction (Figure 6.7). The SH2 domain alone is not sufficient to mediate this binding in vivo and has to be preceded by Shc recruitment to the membrane via the PTB domain. This would explain why the SH2 domain and the ShcΔPTB construct did not significantly localise at the membrane despite their ability to co-precipitate with the receptor. In the cell lysate interaction of the SH2 domain with the FGFR2 is possible, but in the cellular context it is prevented because the SH2 domain is not found in the vicinity of its binding partner(s).

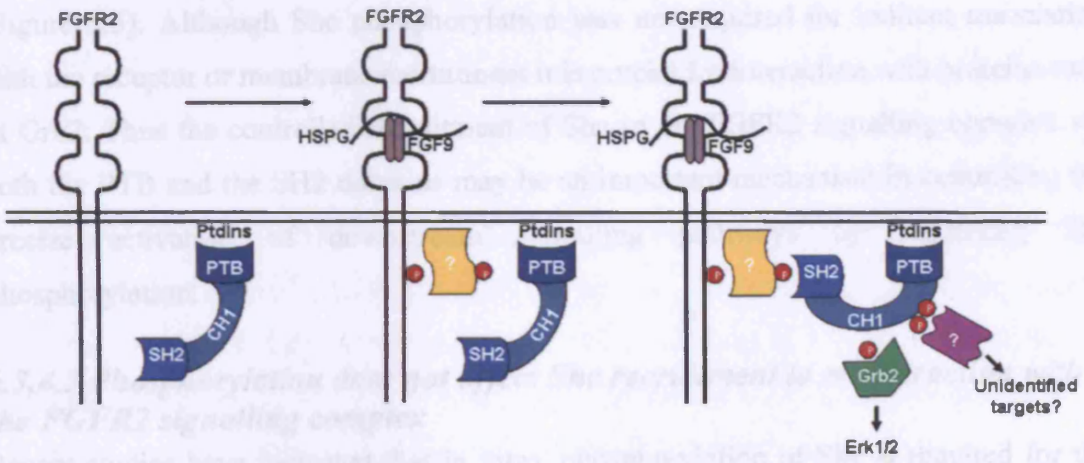

Figure 6.7: Diagrammatic representation of the proposed way in which Shc is recruited to the FGFR2 The Shc PTB domain mediates recruitment to the membrane via interaction with phosphatidylinositol (PtdIns). Activation of the FGFR2 by ligand leads to autophosphorylation and recruitment of signalling proteins. It is unclear whether one or more proteins are important in mediating the interaction of Shc with the FGFR2, but one of them may be the unidentified phospho-protein of 85kDa. Its/their phosphorylation allows interaction with the Shc SH2 domain. Both phosphorylation sites on Shc are able to recruit Grb2 and subsequently Sos, but the Y239/Y240 site may also be involved in recruiting other proteins that activate unknown targets downstream of the FGFR2.

6.3.4.2 The recruitment of Shc to the membrane via the PTB domain may be an important regulator of Shc phosphorylation by the FGFR2

In addition to being important in mediating interactions of the Shc SH2 domain with receptor associated proteins, the recruitment of Shc to the plasma membrane via its PTB domain may also be an important regulator of its phosphorylation. Studies in T cells have revealed that constitutive anchoring of Shc to the membrane via addition of the Ras farnesylation motif leads to its phosphorylation and activation of signalling pathways in the absence of receptor stimulation [297]. Moreover, Shc phosphorylation by c-Src is phosphatidylinositol(4,5)bisphosphate (PIP_2) dependent, whereas phosphorylation by the EGF receptor is PIP_2 independent [298]. The Shc PTB domain

can bind the EGFR directly, which may replace the requirement for PIP_2 binding. This indicates that Shc recruitment to plasma membrane via the PTB domain (where phospholipid or phosphotyrosine binding may occur) might represent an important mechanism in the regulation of Shc phosphorylation and subsequent adaptor protein binding, and could explain how Shc phosphorylation by the FGFR can occur in the absence of all major autophosphorylation sites. It is unclear which changes the expression of the FGFR2 incurs on the plasma membrane environment that would lead to increased Shc localisation to the membrane compared to regular HEK 293T cells (Figure 5.5). Although Shc phosphorylation was not required for indirect association with the receptor or membrane recruitment it is crucial for interaction with proteins such as Grb2. Thus the controlled recruitment of Shc to the FGFR2 signalling complex via both the PTB and the SH2 domains may be an important mechanism in controlling the precise activation of downstream signalling pathways by regulating Shc phosphorylation.

6.3.4.3 Phosphorylation does not affect Shc recruitment to or interaction with the FGFR2 signalling complex

Recent studies have indicated that in vitro, phosphorylation of Shc is required for the SH2 domain to open up and bind tyrosine phosphorylated ligand ([299], in submission). Such a model would add an additional layer of regulation to protein recruitment to RTKs and activation of downstream signalling pathways. One could imagine Shc becoming membrane localised via PTB domain interactions with the membrane in the absence of phosphorylation. Phosphorylation would then be required for it to not only allow Grb2/Sos recruitment but also to stabilise its interaction with activated receptors and other signalling proteins via the SH2 domain. However, in the system investigated in this study, unphosphorylated Shc (Shc3F or Shc in the presence of genistein) co-precipitated and co-localised equally well with the FGFR2. This indicates that the model based on observations made in vitro does not seem to hold true for this particular system.

An explanation for the fact that Shc3F behaves in the same way as full-length Shc could be that in vitro its regulation is very much "black and white", i.e. the SH2 domain is either closed (in the unphosphorylated protein) or open (in the phosphorylated state) for ligand binding. In contrast, in vivo Shc may have the ability to bind some proteins via the SH2 domain in the absence of tyrosine phosphorylation under certain conditions.

This hypothesis was supported by results obtained from pulldown experiments carried out in other cellular systems (data not shown). In vivo, factors in addition to Shc phosphorylation may be involved in regulating the ability of Shc to bind ligands via the SH2 domain. For example, the binding of the PTB domain to the plasma membrane or a PTB binding site on a receptor such as the EGFR may already initiate transition into an open conformation in the absence of Shc phosphorylation. Phosphorylation events in the CH1 domain might subsequently stabilise the open conformation so that binding of ligands via the SH2 domain is prolonged and more stable.

Overall, the data presented in this work indicate a role for the Shc PTB domain in membrane recruitment and for the SH2 domain in binding to FGFR2-interacting proteins (Figure 6.7). This dual binding event is required to ensure correct protein localisation and recruitment to, and binding within the signalling complex formed downstream of the FGFR2. In the absence of one of these domains, these highly regulated events are disrupted and the correct recruitment of Shc to the receptor is prevented. In the absence of the PTB domain, Shc cannot co-localise with the receptor (i.e. is not recruited correctly) (Figure 6.3A and D), whereas the absence of the SH2 domain prevents stable, indirect interaction with the FGFR2 (Figures 6.4 and 6.5). Since full-length Shc constructs co-precipitated more efficiently with the receptor than any individual domains or deletion constructs, it seems that additional complexity and specificity is added to protein interactions involving Shc by the presence of all three domains. Although Shc phosphorylation did not affect recruitment or binding to the FGFR2 ternary complex, this does not rule out a contribution of the CH1 domain in correct positioning of the domains to allows interaction with the membrane and other proteins in a signalling complex induced by FGFR2 activation.

6.3.5 Shc recruitment to the FGFR2 is mediated via indirect association and formation of multiprotein signalling complexes

The absence of direct protein-protein interaction between Shc and FGFR2 indicates that co-precipitation must occur via formation of ternary complexes. Co-precipitation could also take place if Shc was bound to a protein that either directly interacted with the receptor or was found in a complex surrounding the receptor. Thus based on the model described, it seems to be the Shc SH2 domain that interacts with proteins to form a signalling complex surrounding the FGFR2.

Identification of Shc binding partners mediating its recruitment to the FGFR2 is crucial to clearly understand the role of individual Shc domains in FGFR signalling in general. Shc has been shown to bind to various signalling proteins such as for example Gab2 [197], c-Cbl [196], SHIP1, an unidentified protein p145 (downstream of FGFR1 [104]) and others, some of which may be involved in FGFR signalling and could therefore be target proteins for the recruitment of Shc into a signalling complex including this receptor. A tyrosine phosphorylated protein of approximately 40 kDa was observed in anti-Shc and anti-GFP (i.e. anti-FGFR2) immunoprecipitates, and could be a candidate protein for mediating interaction between the two proteins (Figure 5.3A). Another candidate for Shc recruitment to the FGFR2 signalling complex is c-Cbl. Shc has been shown to interact with c-Cbl via its SH2 domain, and a 120kDa phosphoprotein was observed in anti-Shc IPs HEK 293T cells and after longer exposure of the blot in PC12 cells (Figure 5.3). The 85kDa unidentified protein described in Chapter 5 may also be involved in mediating interaction of Shc with the FGFR2. A tyrosine phosphorylated protein of similar molecular weight was observed in the GST-SH2 domain pulldown (Figure 6.5). Therefore this protein may be a likely candidate to mediate the indirect interaction between the FGFR2 and Shc via its SH2 domain. Few proteins have been described that bind to the FGFR, and the number of proteins shown to bind the Shc SH2 domain are also limited. It is thus difficult to indicate potential proteins mediating recruitment of Shc to the FGFR2. The adaptor protein Grb14, which has been shown to bind directly to the FGFR1 [290], has recently been shown to possess an NPXY motif that leads to interaction with IRS-1 in a phosphorylation-independent manner [300]. An interaction of this motif with the Shc PTB domain has not been indicated to date, but would present a possibility of mediating the indirect interaction of Shc with the FGFR2. However, since the SH2 domain was found to mediate interaction with the FGFR2, the interaction of the Shc PTB domain with Grb14 does not seem to play a role in Shc recruitment to this receptor. Altogether, it seems that Shc is recruited to a fairly large protein complex that connects it to the FGFR2. Further work is required to identify Shc-interacting proteins in FGFR signalling as well as the exact role of Shc in the signalling complex formed upon FGFR activation.

One of the functions of Shc downstream of the FGFR (and many other RTKs) is recruitment of the Grb2/Sos complex to the plasma membrane. However, it may also be involved in signalling pathways other than the Erk1/2 pathway. Various reports have

implicated Shc with a role in signalling from activated tyrosine kinase receptors that is independent of mitogenic signalling (i.e. Grb2 recruitment and Erk1/2 activation). The Y239/Y240 phosphorylation site has been shown to be important in signalling to c-*myc* and to have the ability to interact with proteins other than Grb2 in vitro [198, 199, 301, 302]. A mutant Shc protein with a CH1 domain deletion (which still retained Y239/Y240, but was only minimally phosphorylated on these sites and did not bind Grb2) did not affect activation of the Erk1/2 pathway but interfered with transformation as a result of ErbB2 overexpression [200]. These observations indicate that Shc may also play an important role in various other signalling processes that are distinct from its role as a Grb2 recruiting adaptor protein (Figure 6.7). Since FRS2 is primarily involved in Grb2 recruitment [47], different functions of Shc may be of particular interest in FGFR signalling. It would therefore be particularly interesting to determine the proteins that mediate Shc interaction with the FGFR2 as well as proteins that may bind to the two phosphorylated sites in the CH1 domain and may be important in regulation of secondary pathways (other than Erk1/2 activation). The assembly of a specific complex in the absence of direct interaction with the FGFR2 may be important in regulating the specific and precise activation of such targets in response to activation of this receptor compared to other RTKs.

6.3.6 Regulation of signalling specificity by the absence of direct interaction between Shc and the FGFR2

In conclusion, the findings presented in this chapter present a detailed study into the way in which Shc is recruited to activated FGFRs. Although the FGFR2 was used, and differences may exist in terms of the interaction of Shc with other members of the FGFR family, the lack of studies reporting interaction of Shc with any FGFR indicate that these results may be transferable between FGFR1-4. It interesting to note that in the case of signalling from the FGFR, direct association of Shc with the receptor is not required for its functionality. The data thus indicate that different receptor systems are able to discriminate activation of various pathways even if potential binding sites for a number of proteins exist. For example, the FGFR possesses potential Shc SH2 and PTB domain binding sites, but through tight regulation of the order and the stoichiometry of receptor phosphorylation [15], direct interaction of Shc with the receptor can be prevented. Previous work implemented FRS2 as the major component mediating Grb2/Sos recruitment. This indicates that regulation of which signalling complexes are

formed in response to various activated receptor already occurs at the level of which proteins are able to bind the receptor in the cellular context. Thus although potential binding sites exist on receptors, prevention of direct binding of adaptor protein such as Shc or FRS2 leads to formation of different signalling complexes and therefore may play an important role in regulation of downstream signalling pathways. Prevention of binding may be regulated by various mechanisms such as lack of a binding site, structural obstruction of access to a site, lack of phosphorylation of the tyrosine residue required for tight binding or competition with another protein for the same or a nearby site. Furthermore, the work presented in this thesis indicates that although Shc is involved in Grb2 recruitment to the FGFR, it may also carry out other roles in response to FGFR activation. This indicates the requirement for further studies elucidating the proteins that are able to interact with the receptor as well as Shc following FGF stimulation and the exact cellular roles that Shc carries out in response to FGFR activation. Further, this emphasizes that the differential recruitment and/or phosphorylation of proteins by various receptors may play an important role in determining the exact functions they carry out.

Chapter 7

Summary

7.1 Conclusions

The aim of this work was to use two different model systems to investigate differences in protein recruitment to various tyrosine kinase receptors and the role they play in generating signalling specificity. PC12 cells were used as a well-characterised model system to explore how the assembly of different complexes downstream of the EGFR, FGFR and TrkA affects the signal generated by each receptor despite recruitment of the same signalling proteins. The Apert syndrome mutations in FGFR2 were used to create a model system to investigate the effects that receptor extracellular domain mutations have on protein recruitment and generation of specific signals. Additionally, the FGFR2 was used to investigate the interaction of this RTK with Shc and whether differences in the ability of proteins to bind to various receptors affects their involvement in protein complexes and thus the downstream signal initiated. The investigations revealed three main findings regarding signals initiated from various activated RTKs.

Firstly, intrinsic differences within receptor systems were found to regulate signalling specificity. The presence of different binding sites on each receptor can be regulated by the amino acid sequence or by differential and highly controlled phosphorylation. In this way, recruitment of proteins can be controlled, as was observed in the case of the EGFR, FGFR and TrkA. For example, the presence of different binding sites for FRS2 on the three receptors seemed to determine the way in which this protein is involved (i.e. the level of its phosphorylation and subsequently the amounts and types of proteins it can bind). Similarly, it was found that the FGFR2 does not interact with Shc directly. Although other FGFRs may portray a different behaviour, this finding also indicates that the direct binding of a protein to a receptor may regulate its involvement in signalling from this RTK family. The fact that Shc is unable to bind directly to the FGFR correlates with the lower levels of Shc phosphorylation observed in response to FGF compared to EGF stimulation. Using the PC12 model system and FLIM analysis of the interaction between Shc and FGFR2 it was shown that the recruitment of proteins is regulated by intrinsic differences in the receptors. Additional factors such as the occupation of binding sites by other proteins that may thereby form the basis of different signalling complexes may also play an important role in regulation of the exact involvement of specific proteins in signalling from any given RTK. However, the differences observed could be a result of intrinsic variation in the intracellular regions of

the different receptors and were found to be an important regulator of signal specificity and integrity.

The second conclusion that could be drawn from the data presented was that integrity of the receptor system is essential for the generation of correct and specific signals. Mutations affecting the interaction between receptor and ligand are able to disturb this highly regulated system and the normal cellular responses to receptor engagement. Using the Apert syndrome mutations in the FGFR2 as a model system, it was shown that even small changes in the extracellular domain are able to affect the recruitment of signalling proteins to, and the integrity of signals initiated from RTKs. The Apert syndrome mutant FGFR2s exhibit increased affinity for FGF ligand. It was found that the recruitment of adaptor proteins such as Shc, FRS2 and Grb2 as well as activation of the Erk1/2 pathway were grossly affected despite the mutations being removed from the site of protein binding/recruitment. This indicates that even a small disturbance of the signalling system can grossly affect the highly regulated pattern of protein recruitment that is required for the generation of specific signals by different receptors.

Finally, analysis of the changes in receptor glycosylation, localisation and phosphorylation in the presence of the Apert syndrome mutations revealed that various different factors need to be tightly controlled to allow activation of the correct downstream signals. The intrinsic signalling specificity of each type of receptor may not only rely on intracellular properties such as binding sites for protein recruitment, but the overall integrity of the receptor including the extracellular region may be a major determinant of signalling specificity. Receptor glycosylation, localisation and interaction with ligands (both FGFs and HSPGs) were found to be altered in the Apert syndrome mutants. These factors may affect receptor structure and positioning in a functional dimer and are likely to affect receptor phosphorylation. Thereby the combination of these factors contributes to the regulation of cellular responses to stimulation of a particular receptor. Changes in one or more of these properties, as observed in the case of the Apert syndrome mutations in the FGFR2, may therefore have detrimental effects on the integrity of the signals initiated. This work has indicated that all of these factors may contribute to altered recruitment of proteins to a receptor and thereby interfere with correct (and specific) activation of downstream signaling pathways.

In conclusion, this work presents evidence to support the idea that specificity in cell signalling is controlled by a number of different factors. In conjunction, these factors affect protein recruitment, the type, strength, and longevity of the signal. Mutations that lead to alteration of some of these factors can grossly affect the signal generated and its effects on cellular responses. Recruitment of different proteins to RTKs and tight regulation of this process plays a major role in generating signalling specificity. It was shown that this aspect is partly controlled by intrinsic differences between RTKs, although a number of factors were found to affect it. The specific recruitment of proteins into unique multiprotein complexes allows cells to differentiate between the activation of different pathways, which is an important aspect in the regulation of cellular responses to different extracellular stimuli.

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